DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 16063007 Document Date: 06/15/2018

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

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- Drawing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

Takeda EX1104

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 16063007 Document Date: 06/15/2018

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371		Attorney Docket No. I0422.70125US00
		U.S. Application No. (if known, see 37 CFR 1.5) Not Yet Assigned
International Application No. PCT/EP2016/082664	International Filing Date 23 December 2016	Priority Date Claimed 23 December 2015
Title of Invention ZIKA VIRUS VACCINE		
First Named Inventor Jana Barbero Calzado		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). NOTE: The express request under 35 U.S.C. 371(f) will not be effective unless the requirements under 35 U.S.C. 371(c)(1), (2), and (4) for payment of the basic national fee, copy of the International Application and English translation thereof (if required), and the oath or declaration of the inventor(s) have been received.

2. ☐ A copy of the International Application (35 U.S.C. 371(c)(2)) is attached hereto (not required if the International Application was previously communicated by the International Bureau or was filed in the United States Receiving Office (RO/US)).

3. An English language translation of the International Application (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).

4. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))
   a. ☐ is attached.
   b. ☐ was previously filed in the international phase under PCT Rule 4.17(iv).

Items 5 to 8 below concern amendments made in the international phase.

PCT Article 19 and 34 amendments

5. ☐ Amendments to the claims under PCT Article 19 are attached (not required if communicated by the International Bureau) (35 U.S.C. 371(c)(3)).

6. ☐ English translation of the PCT Article 19 amendment is attached (35 U.S.C. 371(c)(3)).

7. ☐ English translation of annexes (Article 19 and/or 34 amendments only) of the International Preliminary Examination Report is attached (35 U.S.C. 371(c)(5)).

Cancellation of amendments made in the international phase

8a. ☐ Do not enter the amendment made in the international phase under PCT Article 19.

8b. ☐ Do not enter the amendment made in the international phase under PCT Article 34.

NOTE: A proper amendment made in English under Article 19 or 34 will be entered in the U.S. national phase application absent a clear instruction from applicant not to enter the amendment(s).

The following items 9 to 17 concern a document(s) or information included.

9. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

10. ☒ A preliminary amendment.

11. ☒ An Application Data Sheet under 37 CFR 1.76.

12. ☐ A substitute specification. NOTE: A substitute specification cannot include claims. See 37 CFR 1.125(b).

13. ☐ A power of attorney and/or change of address letter.

14. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.3 and 37 CFR 1.821-1.825.

15. ☐ Assignment papers *(cover sheet and document(s)).* Name of Assignee: _____

16. ☐ 37 CFR 3.73(c) Statement *(when there is an Assignee).*

U.S. APPLN. No. (if known – see 37 CFR 1.5)	INTERNATIONAL APPLICATION No.	ATTORNEY DOCKET No.
Not Yet Assigned	PCT/EP2016/082664	I0422.70125US00

17. [X] Other items or information: International PCT Publication
PCT/IB/301
PCT/IB/304
International Search Report
Written Opinion

The following fees have been submitted.	CALCULATIONS	PTO USE ONLY
18. [X] Basic national fee (37 CFR 1.492(a)) **$300**	$ 300.00	
19. [X] Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... **$0** All other situations **$760**	$ 760.00	
20. [X] Search fee (37 CFR 1.492(b)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... **$0** Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority ..... **$140** International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB ..... **$520** All other situations **$660**	$ 520.00	
TOTAL OF 18, 19, and 20 =	$ 1,580.00	

[X] Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) in an electronic medium or computer program listing in an electronic medium) (37 CFR 1.492(j)).
Fee for each additional 50 sheets of paper or fraction thereof **$400**

Total Sheets	Extra Sheets	Number of each addition 50 or fraction thereof (round **up** to a whole number)	RATE	
127 - 100 =	27 / 50 =	1	x **$400**	$ 400.00

Surcharge of **$140.00** for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =	0	x **$100**	$
Independent claims	- 3 =	0	x **$460**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$820**	$

Processing fee of **$140.00** for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i)). +	$
TOTAL OF ABOVE CALCULATIONS =	$ 1,980.00
[ ] **Applicant asserts small entity status.** See 37 CFR 1.27. Fees above are reduced by ½.	
[ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Fees above are reduced by ¾. Applicant must attach form PTO/SB/15A or B or equivalent.	
TOTAL NATIONAL FEE =	$ 1,980.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$50.00** per property. +	$
TOTAL FEES ENCLOSED =	$ 1,980.00
Amount to be refunded:	$
Amount to be charged:	$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 23/2825 in the amount of $ _____ to cover the above fees.

c. ☒ The Director is hereby authorized to charge additional fees which may be required, or credit any overpayment, to Deposit Account No. 23/2825 as follows:

i. ☐ any required fee.

ii. ☒ any required fee except for excess claims fees required under 37 CFR 1.492(d) and (e) and multiple dependent claim fee required under 37 CFR 1.492(f).

d. ☒ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038. The PTO-2038 should only be mailed or faxed to the USPTO. However, when paying the basic national fee, the PTO-2038 may NOT be faxed to the USPTO.

ADVISORY: If filing by EFS-Web, do **NOT** attach the PTO-2038 form as a PDF along with your EFS-Web submission. Please be advised that this is **not** recommended and by doing so your **credit card information may be displayed via PAIR**. To protect your information, it is recommended to pay fees online by using the electronic payment method.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File)Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013, and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE 1: By providing this statement under 37 CFR 1.55 or 1.78, **this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA**.

NOTE 2: A U.S. national stage application may not claim priority to the international application of which it is the national phase. The filing date of a U.S. national stage application is the international filing date. See 35 U.S.C. 363.

Correspondence Address

☒ The address associated with Customer Number: 23628 **OR** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone			
Email					

Signature	/John R. Van Amsterdam/	Date	June 15, 2018
Name (Print/Type)	John R. Van Amsterdam	Registration No. (Attorney/Agent)	40,212

Application Data Sheet

Inventor Information

Inventor Number::	1
Given Name::	Jana
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Country of Residence::	Austria
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City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1050

Inventor Number::	2
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Family Name::	Nebenführ
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City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1030

Inventor Number::	3

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City of Residence::	Siegenfeld
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Street of mailing address::	Rosenthal 63
City of mailing address::	Siegenfeld
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Inventor Number::	4
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Street of mailing address::	Rechte Wienzeile 249/15
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Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1120

Inventor Number::	5
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Family Name::	Wruss
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Street of mailing address::	Dampfgasse 25/27-29
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1100

Correspondence Information

Correspondence Customer Number::	23628

Application Information

Application Type::	Regular
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	Yes
Computer Readable Form (CRF)?::	Text File
Title::	ZIKA VIRUS VACCINE
Attorney Docket Number::	I0422.70125US00
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	23
Small Entity?::	No
Petition included?::	No
Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy	No

Order pursuant to 37 CFR 5.2::

Representative Information

Representative Customer Number:: 23628

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::	Prior Appl Status::
This Application	a 371 of international	PCT/EP2016/082664	12/23/16	

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::	DAS Access::
European Patent Office	16182845.4	08/04/16	Yes	
European Patent Office	16176049.1	06/23/16	Yes	
European Patent Office	16176025.1	06/23/16	Yes	
European Patent Office	16161068.8	03/18/16	Yes	
European Patent Office	15202585.4	12/23/15	Yes	

Applicant Information

Applicant Number:: 1

Applicant Type:: Assignee

Organization Name:: Valneva Austria GmbH

Street of mailing address:: Campus Vienna Biocenter 3

City of mailing address:: Vienna

Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1030

Assignee Information Including Non-Applicant Assignee Information

Assignee Number::	1
Organization Name::	Valneva Austria GmbH
Street of mailing address::	Campus Vienna Biocenter 3
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1030

Authorization or Opt-Out of Authorization to Permit Access

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).** This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants. See 37 CFR 1.4(d) for the manner of making signatures and certifications.			
Signature	/John R. Van Amsterdam/	Date (YYYY-MM-DD)	2018-06-15
Name	John R. Van Amsterdam	Registration Number	40,212

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

To:

DEMPSTER, Robert
Maschio & Soames IP Limited
30 Carlton Crescent
Southampton Hampshire SO15 2EW
ROYAUME-UNI

Date of mailing *(day/month/year)*	IMPORTANT NOTIFICATION
30 January 2017 (30.01.2017)	
Applicant's or agent's file reference	International application No.
P5706PC00	PCT/EP2016/082664

The applicant is hereby **notified** that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

VALNEVA SE (all designated States)

International filing date: 23 December 2016 (23.12.2016)

Priority date(s) claimed: 23 December 2015 (23.12.2015)
18 March 2016 (18.03.2016)
23 June 2016 (23.06.2016)
23 June 2016 (23.06.2016)
04 August 2016 (04.08.2016)

Date of receipt of the record copy by the International Bureau: 27 January 2017 (27.01.2017)

List of designated Offices:

AP: BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW
EA: AM, AZ, BY, KG, KZ, RU, TJ, TM
EP: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR
OA: BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG
National: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW

ATTENTION: The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau. **In addition, the applicant's attention is drawn to:**

- time limits for entry into the national phase (see www.wipo.int/pct/en/texts/time_limits.htmland *PCT Applicant's Guide*, National Phase, especially Chapters 3 and 4)
- requirements regarding priority documents (if applicable) (see *PCT Applicant's Guide*, International Phase, paragraph 5.070)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 89 75

Authorized officer
Campin Helene
e-mail pct.team5@wipo.int
Telephone No. +41 22 338 74 05

NOTIFICATION OF RECEIPT OF RECORD COPY

Date of mailing *(day/month/year)*
30 January 2017 (30.01.2017)

A copy of this notification is being sent to the receiving Office and to the International Searching Authority.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION, OBTENTION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

DEMPSTER, Robert
Maschio & Soames IP Limited
30 Carlton Crescent
Southampton Hampshire SO15 2EW
ROYAUME-UNI

Date of mailing *(day/month/year)* 02 February 2017 (02.02.2017)	
Applicant's or agent's file reference P5706PC00	**IMPORTANT NOTIFICATION**
International application No. PCT/EP2016/082664	International filing date *(day/month/year)* 23 December 2016 (23.12.2016)
International publication date *(day/month/year)* **Not yet published**	Priority date *(day/month/year)* 23 December 2015 (23.12.2015)
Applicant VALNEVA AUSTRIA GMBH	

The applicant is hereby notified of the date of receipt (or of obtaining by the International Bureau) of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to the date of receipt, **the priority document concerned was submitted or transmitted to or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-*bis*)**. This Form replaces any previously issued notification concerning submission, transmittal or obtaining of priority documents.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
23 December 2015 (23.12.2015)	15202585.4	EP	31 January 2017 (31.01.2017)
18 March 2016 (18.03.2016)	16161068.8	EP	31 January 2017 (31.01.2017)
23 June 2016 (23.06.2016)	16176025.1	EP	31 January 2017 (31.01.2017)
23 June 2016 (23.06.2016)	16176049.1	EP	31 January 2017 (31.01.2017)
04 August 2016 (04.08.2016)	16182845.4	EP	NR

The letters "NR" denote a priority document which, on the date of mailing of this Form, had not yet been received or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-*bis*). Where the applicant has failed to either submit, request to prepare and transmit, or to request the International Bureau to obtain the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

An asterisk "*" next to a date of receipt, denotes a priority document submitted or transmitted to or obtained by the International Bureau but not in compliance with Rule 17.1(a), (b) or (b-*bis*) (the priority document was received after the time limit prescribed in Rule 17.1(a); the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b) or the request to the International Bureau to obtain the priority document was made after the applicable time limit under Rule 17.1(b-*bis*)). Even though the priority document was not furnished in compliance with Rule 17.1(a), (b) or (b-*bis*), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 89 75

Authorized officer
Nora Lindner
e-mail pct.team5@wipo.int
Telephone No. +41 22 338 74 05

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
P5706PC00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP2016/082664	23 December 2016 (23-12-2016)	23 December 2015 (23-12-2015)
Applicant		
VALNEVA AUSTRIA GMBH		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [X] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/082664

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/082664

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 C07K14/005
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	B. D. COX ET AL: "Predicting Zika virus structural biology: Challenges and opportunities for intervention", ANTIVIRAL CHEMISTRY & CHEMOTHERAPY., vol. 24, no. 3-4, 1 August 2015 (2015-08-01), pages 118-126, XP055328128, GB ISSN: 0956-3202, DOI: 10.1177/2040206616653873 the whole document ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2017	10/04/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lewis, Birgit

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987, ISSN: 0264-410X [retrieved on 2001-08-14] cited in the application the whole document	1-17
Y	US 8 765 148 B2 (WIZEL BENJAMIN [AT] ET AL) 1 July 2014 (2014-07-01) cited in the application column 1	13,14
X,P	WO 2016/145149 A1 (THE US SECRETARY OF THE ARMY ON BEHALF OF THE WALTER REED ARMY INST OF) 15 September 2016 (2016-09-15) page 5, lines 8-17 paragraphs [0037] - [0040] paragraph [0048] claims 1-19	1-12,15
E	WO 2017/009873 A1 (BHARAT BIOTECH INT LTD) 19 January 2017 (2017-01-19) examples 1, 2,3,5-7	1-12,15
A	US 6 309 650 B1 (KIM HYUN SU ET AL) 30 October 2001 (2001-10-30) cited in the application examples 3-5; tables 5,6	1-17
X,P	CN 105 749 268 A (SINOVAC RES & DEV CO LTD) 13 July 2016 (2016-07-13) the whole document	1-8, 10-12,15
X,P	RAFAEL A. LAROCCA ET AL: "Vaccine protection against Zika virus from Brazil", NATURE, vol. 536, no. 7617, 28 June 2016 (2016-06-28), pages 474-478, XP055314483, United Kingdom ISSN: 0028-0836, DOI: 10.1038/nature18952 cited in the application abstract page 475, right-hand column, last paragraph - page 476, left-hand column, paragraph 1	1-12, 15-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/082664

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 8765148 B2	01-07-2014	EP 2536427 A1	26-12-2012
		US 2012308618 A1	06-12-2012
		US 2015216967 A1	06-08-2015
		WO 2011101465 A1	25-08-2011
WO 2016145149 A1	15-09-2016	NONE	
WO 2017009873 A1	19-01-2017	US 2017014502 A1	19-01-2017
		WO 2017009873 A1	19-01-2017
US 6309650 B1	30-10-2001	AU 748730 B2	13-06-2002
		CA 2301000 A1	11-03-1999
		CN 1272879 A	08-11-2000
		DE 69837211 T2	06-12-2007
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		DK 1604685 T3	18-06-2012
		EP 1025209 A1	09-08-2000
		EP 1604685 A2	14-12-2005
		ES 2281929 T3	01-10-2007
		ES 2382946 T3	14-06-2012
		JP 4161017 B2	08-10-2008
		JP 2001514844 A	18-09-2001
		MY 129773 A	30-04-2007
		NL 300391 I1	03-08-2009
		NZ 503522 A	29-06-2001
		US 6309650 B1	30-10-2001
		WO 9911762 A1	11-03-1999
CN 105749268 A	13-07-2016	NONE	

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2016/082664	23.12.2016	23.12.2015

International Patent Classification (IPC) or both national classification and IPC
INV. A61K39/12 C07K14/005

Applicant
VALNEVA AUSTRIA GMBH

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Lewis, Birgit Telephone No. +49 89 2399-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☐ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

   see separate sheet

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-17
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item II

Priority

The present application claims 5 priorities.

The first priority (23.12.2015) is not valid because patent application EP15202585 does not relate to Zika virus at all.

The second priority is dated 18.03.2016. EP16161068 relates to Zika virus including an inactivated Zika virus but not to a genome of Zika strain H/PF/2013 (SEQ ID NO: 13 of the present application, accession number KJ776791.1). Example 2 discloses virus growth and purification including a concentration step by chilling in ice for 2 hours after adding 0.5-2mg of protamine sulfate and ultracentrifugation on sucrose gradients at 38.000 g for 18 hours followed by filtration through a 0.22 µm filter. Example 3 relates to virus inactivation by formaldehyde (0.018% at 22°c or 4°C). Example 4 tests the immunogenicity of the virus with aluminium hydroxyde adjuvant but does not show the outcome with regard to % seroprotection. From this concludes that the present set of claims (claims 1-15) can only partially claim this priority, i.e. only when SEQ ID NOs. are referred to that are disclosed in EP16161068. Finally, it is noted that EP16161068 does not relate to the subject-matter of present claims 16 and 17 (relating to '*PS at amounts too low to detect*'). Thus, claims 16 and 17 are not entitled to the priority of 18.03.2016.

The third and fourth priorities were both filed on 23-06-2016.

EP16176025 (PRIO3) is the first disclosure of the idea to use the successful JEV production system IXARIO® as basis for the production of Zika virus. This includes the use of protamine sulfate to remove contaminating DNA and surprisingly as well immature and non-infectious virus particles. The aspect of a purity with '*PS at amounts too low to detect*' is not explicitly mentioned. Therefore, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 23-06-2016. PRIO3 also mentions for the first time the Zika strain H/PF/2013 (SEQ ID NO: 13 of the present application, SEQ ID NO: 13 in PRIO3). In order to demonstrate or confirm complete entitlement of present claims 1-15 to claim this priority, the applicant will be asked to identify for each SEQ ID NO: in the claims, the corresponding sequence in EP16176025. If this is not possible, this priority can only be partially claimed.

EP16176049 (PRIO4) deals with the purification of viruses, the examples being directed at Flaviviridae, Zika virus being one of them. Example 2 addresses virus purity after PS treatment. However, it does not relate to the subject-matter of present claims 16 and 17 (relating to '*PS at amounts too low to detect*'). Thus, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 23-06-2016.

EP16182845 (PRIO5) of 04-08-2016 addresses the purity of the vaccine with regard to protamine salt and the introduction of an improved sucrose gradient. It will need to be discussed if the disclosures made in EP16182845 are sufficient to entitle the application to the priority date of 04-08-2016. At the moment, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 04-08-2016.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1	B. D. COX ET AL: "Predicting Zika virus structural biology: Challenges and opportunities for intervention", ANTIVIRAL CHEMISTRY & CHEMOTHERAPY., vol. 24, no. 3-4, 1 August 2015 (2015-08-01), pages 118-126, XP055328128
D2	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987
D3	US 8 765 148 B2 (WIZEL BENJAMIN ET AL) 1 July 2014 (2014-07-01) cited in the application

D1 is regarded as being the prior art closest to the subject-matter of claim 1, and discloses that Zika virus is in the center of an emerging crisis that requires the development of a vaccine. D1 does not relate to an inactivated Zika virus vaccine. Hence, claims 1 and dependent and related claims 2-17 are new.

The problem to be solved by the present invention may be regarded as the provision of a safe and efficient Zika virus vaccine.

The solution to this problem proposed in claim 1 of the present application, namely an inactivated Zika virus vaccine that is able to confer seroprotection on at least 70% of subjects is not considered as involving an inventive step because from D1 it was already known that Zika virus belongs to the genus Flavivirus, like DENV or JEV (D1: p. 119, left col. §2). From D2 it was further known, how to produce a successful Flavivirus vaccine. Hence it is considered that the skilled person looking to develop a

Zika virus vaccine would turn to D2 to find a strategy for the development of a Flavivirus vaccine with a reasonable expectation of success and without the exercise of inventive skill, in order to solve the problem posed.

Having regard to the dependent and related claims, it appears that none of the claims relates to features that are not readily derivable from either document D1 or D2 or document D3 (which discloses the specific adjuvants of claims 13 and 14). This includes at least some of the sequences listed in claims 3 and 4 (note that D1 already states that many Zika virus sequences are known). Finally, it is noted that the sequences of claims 3 and 4 are not shown in the application as having any surprising effect with regard to the development of a vaccine. Hence, they are obvious selections that become available to the skilled person as soon as a new strain is identified and published, and cannot establish an inventive step.

Re Item VI

Certain documents cited

D4	WO 2016/145149 A1 (THE US SECRETARY OF THE ARMY ON BEHALF OF THE WALTER REED ARMY INST OF) 15 September 2016 (2016-09-15)
D5	WO 2017/009873 A1 (BHARAT BIOTECH INT LTD) 19 January 2017 (2017-01-19)
D6	CN 105 749 268 A (SINOVAC RES & DEV CO LTD) 13 July 2016 (2016-07-13)
D7	RAFAEL A. LAROCCA ET AL: "Vaccine protection against Zika virus from Brazil", NATURE, vol. 536, no. 7617, 28 June 2016 (2016-06-28), pages 474-478, XP055314483

WO 2016/145149 (D4) published on 15 September 2016 claims the priority of 11-03-2015. Its priority is, thus before the first priority of the present application.

It discloses a vaccine comprising at least on inactivated Dengue virus and one inactivated non-Dengue virus, for example Zika virus. The Flavivirus is grown in Vero cells, treated with protamine sulfate (2mg/ml), purified over a sucrose gradient and activated by formaldehyde treatment (5mM formalin at 22°C for 10 days). The vaccine is formulated with aluminium hydroxyde as adjuvant.

WO 2017/009873 (D5) published on 19 January 2017 claims the priority of 16 July 2015. Its priority is, thus before the first priority of the present application.

It discloses a vaccine comprising an inactivated Zika virus, f. ex. formaldehyde deactivated.

CN 105 749 268 (D6) published on 13 July 2016 claims the priority of 11 April 2016. Its priority is, thus before the priorities PRIO3 and PRIO4 of the present application.

It discloses an inactivated (f. ex. formaldehyde inactivated) Zika virus vaccine. Purification is done by sucrose gradient centrifugation and the vaccine may be formulated with an aluminium salt.

Larocca et al. (D7) published on 28 June 2016, discloses an inactivated Zika virus vaccine (PIV) which shows complete protection after one single administration in a mouse model. The strain used was Puerto Rican PRVABC59 Zika virus which is SEQ ID NO: 4 in the present application, it was passaged in Vero cells, formalin inactivated (0.05%, 22°C for 7 days) and formulated with alum.

Re Item VIII

Certain observations on the international application

Claim 1 does not meet the requirements of Article 6 PCT because the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved ('*seroprotection on at least 70% of subjects*'), which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result.

It is clear from the description and the examples, that the advantageous immunogenicity and safety of the inactivated Zika virus vaccine lies in the preparation and purification and not in the strain selection or the corresponding genomes and E proteins. Hence, dependent claims 2-4 cannot provide features to overcome the clarity objection concerning claim 1.

Dependent claims 5-9 address the inactivation step. The respective features ('*inactivated by* ...'), however, relate to a method and cannot *per se* serve to characterize a product. Hence, also claims 5-9 are unclear in the sense of Article 6 PCT.

Dependent claims 10-15 relate to the adjuvant and/or excipient. Adjuvants and excipients often have an effect on the performance of a vaccine but they cannot serve to characterize the inactivated virus particle in order to overcome the objection concerning claim 1.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 June 2017 (29.06.2017)

WIPO | PCT

(10) International Publication Number
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A61K 39/12 (2006.01) *C07K 14/005* (2006.01)

(21) **International Application Number**:
PCT/EP2016/082664

(22) **International Filing Date**:
23 December 2016 (23.12.2016)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:

15202585.4	23 December 2015 (23.12.2015)	EP
16161068.8	18 March 2016 (18.03.2016)	EP
16176025.1	23 June 2016 (23.06.2016)	EP
16176049.1	23 June 2016 (23.06.2016)	EP
16182845.4	4 August 2016 (04.08.2016)	EP

(71) **Applicant**: **VALNEVA AUSTRIA GMBH** [AT/AT]; Campus Vienna Biocenter 3, 1030 Vienna (AT).

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/109225 A1

(54) **Title**: ZIKA VIRUS VACCINE

(57) **Abstract**: Described herein are Zika virus vaccines and compositions and methods of producing and administering said vaccines to subjects in need thereof.

ZIKA VIRUS VACCINE

FIELD OF INVENTION

The disclosure relates to methods for the purification of Zika viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate. The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site http://www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including

Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measures involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

A preventative vaccine against Zika virus is a pressing medical need in endemic areas and in geographical areas where the vector is spreading. Furthermore, as Zika infection has dire consequences on embryonic and fetal development, a safe and effective vaccine for women of child-bearing potential or pregnant women is needed. Vaccines administered during pregnancy must be very safe for both the mother and the developing fetus. While live attenuated viral vaccines are highly effective, they are often not considered safe enough for administration to pregnant women. In this regard, inactivated viral vaccines, which lack the ability to propagate in the vaccinated subject, are considered much safer. Development of an inactivated Zika virus vaccine for administration to at-risk patients would fill this need.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can be very efficiently separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to Zika virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely

devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Detail experimental examples to the above are provided for Zika virus.

The herein disclosed in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was unexpectedly higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, Nature doi:10.1038/nature18952.). Inactivated viruses are among the safest vaccines and especially preferred for delivery to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figure 7: Alignment (shading: % identity, aa), E-protein.

Figure 8: An example of virus particle maturation in the host cell. As observed in flaviviruses, full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID50 activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 9: An exemplary downstream virus purification process from the crude harvest to formulation of the drug substance (vaccine), a preferred embodiment of the process of the invention (**A**). A flow-chart of an exemplary virus inactivation process is shown in (**B**).

Figure 10: PS treatment results in selective removal of Zika virus aggregates and Vero HCP and LMW impurities (SEC-HPLC of 30x concentrated Zika Virus harvest day 5).

Figure 11: SEC-HPLC of individual 30x concentrated Zika harvest prior to PS treatment at different time points.

Figure 12: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment at different time points. The smaller graph indicates the observed cytopathic effect (CPE) over time.

Figure 13: Preparation of the sucrose gradient.

Figure 14: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 15: Comparison of JEV and ZikaV harvest schedules/yields.

Figure 16: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column. Both panels are from the same chromatogram. The upper graph is the complete elution profile; the lower graph is an enlargement of the ZIKAV elution peak.

Figure 17: SEC-MALLS analysis of inactivated ZikaV.

Figure 18: Cumulative particle size distribution of Zika NIV.

Figure 19: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 20: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 21: Correlation between JEV antigen content in neutralized inactivated virus (NIV) analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus.

Disclosed herein are downstream processes for purifying Zika virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for

propagation on a particular cell substrate or for a partially / fully cell substrate adapted Zika virus particle.

Aspects of the invention provide processes for the purification of infectious Zika virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the content of residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL.

In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the detection limit of HPLC, in particular below the detection limit in the final drug substance. In some embodiments, the PS content is tested by HPLC or size exclusion chromatography (SEC). For example, HPLC is validated for PS determination in JEV sucrose gradient pool samples as a routine release assay and is very sensitive (i.e., LOQ 3 µg/mL; LOD 1 µg/mL). In the current invention, PS content in in Zika virus DS samples was <LOD. In one embodiment, the HPLC assessment of PS

content can be performed on a Superdex Peptide 10/300GL column (GE: 17-5176-01) using 30% Acetonitrile, 0,1% Trifluoroacetic acid as solvent with a flow rate of 0,6 ml/min at 25°C and detection at 214 nm. A more sensitive method of measurement for residual protamine in a purified virus preparation is mass spectrometry (MS). In some embodiments, the residual PS levels in a Zika virus preparation are tested by MS or other such highly sensitive method, e.g. nuclear magnetic resonance (NMR). With this method, residual PS, as well as fragments and/or break-down products of PS, can be detected at trace amounts, such as levels as low as, for example, 10^6, 10^7 or 10^8 molecules per typical sample load. In some embodiments, the PS levels are tested in the sucrose gradient pool. In some embodiments, the PS levels are tested in the drug product. In some embodiments, the PS levels are tested in the drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of about 100 kDa.

The process according to the current invention may also comprise the use of a sucrose gradient, preferably an optimized sucrose gradient. The sucrose gradient is preferably optimized for the removal of protamine sulfate, also for the removal of immature viral particles or other viral particles which are non-infectious or host cell proteins or nucleic acids (DNA, RNA, mRNA, etc) or other host cell debris. In the current invention the optimized sucrose gradient comprises at least two, at least three, at least four layers of sucrose solutions with different densities. In one embodiment, the virus preparation to be purified is provided in a sucrose solution which has a density of about 8%, about 9%, about 10%, about 11%, about 12% sucrose (w/w), preferably about 10%. In one embodiment, one sucrose solution in the gradient has a density of about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55% sucrose (w/w),

preferably about 50%. In one embodiment, one sucrose solution in the gradient has a density of about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40% sucrose (w/w), preferably about 35%. In one embodiment, one sucrose solution in the gradient has a density of about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20% sucrose (w/w), preferably about 15% sucrose. In a preferred embodiment, the sucrose gradient comprises three layers of sucrose solutions of about 50%, about 35% and about 15% (w/w) sucrose and the virus composition to be purified is contained in about 10% (w/w) sucrose. Because the invention provided for means to not only test for host cell DNA but also immature viral particles, the skilled person in the art is able to more precisely optimize the sucrose gradient for most efficient purification and include additional tools such as PRNT assay to monitor purification success.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may be optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the Zika virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In preferred embodiments, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian

cell line. In some embodiments, said cell line is an EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the Zika virus particles obtainable by any of the processes described herein for treating and/or preventing (i.e. protecting from) a viral infection. In a preferred embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on the average for their age, sex, and ethnicity. This is a result of the brain failing to undergo proper

embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has an RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the process of the invention results in an enrichment of infectious Zika virus particles from the crude harvest comprising infectious Zika virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially at least 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

A unique aspect of the current invention is the realization that know-how related to the vaccine design and purification approach used for the Japanese Encephalitis Vaccine (JEV) IXIARO® (see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) may be employed and improved upon in order to expedite the development of a Zika virus vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, was complemented by further significant improvements disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines is their greatly reduced protamine salt (SEQ ID NO: 1) content in the final drug substance facilitated by the development of an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the Vero (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or the plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently

inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation needs to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0.01 MOI (multiplicity of infection) to allow for viral production prior to passaging. In some embodiments, the Vero cells are incubated for at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. In order to minimize the adaption of Zika virus strain to Vero cells, it was found that Vero cells could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days

2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 μm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the Zika virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant", is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA. The

fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in the experimental part. It was surprisingly found that the addition of a virus-containing fraction with 10% (w/w) sucrose to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) resulted in efficient separation of protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, i.e. a Zika virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agents for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using

such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphobetaine. A preferred inactivation is inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of

time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments, the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time. It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

Any of the methods or uses described herein may be for the prevention of a Zika virus infection in a subject. As used herein, the terms “prevent,” “preventing” and “protection from” include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the Zika virus. As used herein, antigen(s), such as an inactivated Zika virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck, trunk, upper arms; headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject has been previously infected with or vaccinated against Dengue virus; i.e., at risk for antibody-dependent enhancement of disease. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus antibodies). In some embodiments, the therapeutically effective amount is sufficient to provide seroprotection in a subject; i.e., to generate sufficient antigen-specific antibodies to prevent/protect

from infection. In some embodiments, seroprotection is conferred on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects. In some embodiments, seroprotection is defined by a reduction in the number of Zika virus plaques by 50% or more in a plaque reduction neutralization test (PRNT) by a 1:10 or higher dilution of sera from a vaccinated subject. In some embodiments, an effective amount of the Zika vaccine is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the

Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)). Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 71). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its entirety). In some embodiments, the adjuvant is IC31®, i.e. $KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccines to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may

have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having,", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Table 1. Overview of process buffers and stock solutions.

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.

C	25 mM Tris, 150 mM NaCl	7.4±0.2	16.5
D	1 M Tris	7.4±0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
K	10 x PBS	7.4±0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4±0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4±0.2	1.3

Table 2. Abbreviations.

°Bx	Degrees Brix = sugar content (w/w) of an aqueous solution*
BSA	Bovine serum albumin
CC700	Capto™ Core 700
CPE	Cytopathic effect
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
ID	Inner diameter
JEV	Japanese Encephalitis virus
LAL	Limulus amebocyte lysate
LDS buffer	Lithium dodecyl sulfate sample loading buffer
LOD	Limit of detection
LOQ	Limit of quantitation
MALLS	Multiangle light scattering
mAU	Milli absorbance units
MS	Mass spectroscopy
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
PD	Process development
PFU	Plaque forming units
p.i.	Post-infection
PS	Protamine sulphate or protamine sulfate
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin
Rms radius	Root mean square radius
rMSB	Research master seed bank
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SGP	Sucrose gradient purified
SDS	Sodium dodecyl sulphate

TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
WFI	Water for injection
ZikaV	Zika virus

*Degrees Brix (°Bx) is the sugar content of an aqueous solution. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. °Bx corresponds to the sucrose content in percent (w/w), e.g., 45 °Bx equals 45 % (w/w) sucrose.

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Table A. Primers for Zika virus sequencing: lower case letters indicate bases not included in ZIKA but containing restriction sites for later cloning when needed (therefore, two Tms provided).

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
1	9320_Zika_PF_1F	SEQ ID NO: 74 ttaggatccGTTGTTGATCTGTGTGAAT	69.9	74.6	707
	9321_Zika_PF_1R	SEQ ID NO: 75 taactcgagCGTACACAACCCAAGTT	69.3	75.6	
2	9322_Zika_PF_2F	SEQ ID NO: 76 ttaggatccTCACTAGACGTGGGAGTG	70	73.9	704
	9323_Zika_PF_2R	SEQ ID NO: 77 taactcgagAAGCCATGTCYGATATTGAT	69.8	73.7	
3	9324_Zika_PF_3F	SEQ ID NO: 78 ttaggatccGCATACAGCATCAGGTG	72.3	74.5	712
	9325_Zika_PF_3R	SEQ ID NO: 79 taactcgagTGTGGAGTTCCGGTGTCT	72	76.4	
4	9326_Zika_PF_4F	SEQ ID NO: 80 ttaggatccGAATAGAGCGAARGTTGAGATA	70.9	74	712
	9327_Zika_PF_4R	SEQ ID NO: 81 taactcgAGTGGTGGGTGATCTTCTTCT	70.5	73.7	
5	9328_Zika_PF_5F	SEQ ID NO: 82 ttaggatcCAGTCACAGTGGAGGTACAGTAC	70.3	75	704
	9329_Zika_PF_5R	SEQ ID NO: 83 taactcgagCRCAGATACCATCTTCCC	71.5	77.3	
6	9330_Zika_PF_6F	SEQ ID NO: 84 ttaggatCCCTTATGTGCTTGGCCTTAG	70.7	72.7	698
	9331_Zika_PF_6R	SEQ ID NO: 85 taactcgagTCTTCAGCCTCCATGTG	70.4	76.9	
7	9332_Zika_PF_7F	SEQ ID NO: 86 ttaggatccAATGCCCACTCAAACATAGA	71.9	75	716
	9333_Zika_PF_7R	SEQ ID NO: 87 taactcgagTCATTCTCTTCTTCAGCCCTT	71	74	
8	9334_Zika_PF_8F	SEQ ID NO: 88 ttaggatccAAGGGTGATCGAGGAAT	70.9	75.2	703
	9335_Zika_PF_8R	SEQ ID NO: 89 taactcgagTTCCCTTCAGAGAGAGGAGC	71.9	73.4	
9	9336_Zika_PF_9F	SEQ ID NO: 90 ttaggatccTCTTTTGCAAACTGCGATC	71.9	75	699
	9337_Zika_PF_9R	SEQ ID NO: 91 taactcgagTCCAGCTGCAAAGGGTAT	71	74.9	
10	9338_Zika_PF_10F	SEQ ID NO: 92 ttaggatccGTGTGGACATGTACATTGA	71.4	75.8	706
	9339_Zika_PF_10R	SEQ ID NO: 93 taactcgagCCCATTGCCATAAAGTC	70.4	75.8	

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
11	9340_Zika_PF_11F	SEQ ID NO: 94 ttaggatccTCATACTGTGGTCCATGGA	71.6	78.1	692
	9341_Zika_PF_11R	SEQ ID NO: 95 taactcgagGCCCATCTCAACCCTTG	74	78	
12	9342_Zika_PF_12F	SEQ ID NO: 96 ttaggatccTAGAGGGCTTCCAGTGC	70.9	74	707
	9343_Zika_PF_12R	SEQ ID NO: 97 taactcgAGATACTCATCTCCAGGTTTGTTG	70.2	72.2	
13	9344_Zika_PF_13F	SEQ ID NO: 98 ttaggatccGAAAACAAAACATCAAGAGTG	70.6	75.4	726
	9345_Zika_PF_13R	SEQ ID NO: 99 taactcgagGAATCTCTCTGTCATGTGTCCT	71.9	75.6	
14	9346_Zika_PF_14F	SEQ ID NO: 100 ttaggatccTTGATGGCACGACCAAC	73.1	75.6	715
	9347_Zika_PF_14R	SEQ ID NO: 101 ttaggatccGTTGTTGATCTGTGTGAAT	70.8	77.9	
15	9348_Zika_PF_15F	SEQ ID NO: 102 taactcgagCAGGTCAATGTCCATTG	71.9	75.4	719
	9349_Zika_PF_15R	SEQ ID NO: 103 ttaggatccTGTTGTGTTCCTATTGCTGGT	73.9	77.2	
16	9350_Zika_PF_16F	SEQ ID NO: 104 taactcgaGTGATCAGRGCCCCAGC	72.3	75.4	703
	9351_Zika_PF_16R	SEQ ID NO: 105 ttaggatccTGCTGCCCAGAAGAGAA	72	76.3	
17	9352_Zika_PF_17F	SEQ ID NO: 106 taactcgaGCACCAACAYGGGTTCTT	73.6	76	705
	9353_Zika_PF_17R	SEQ ID NO: 107 ttaggatcCTCAAGGACGGTGTGGC	72	75.5	
18	9354_Zika_PF_18F	SEQ ID NO: 108 taactcgagCAATGATCTTCATGTTGGG	71.7	75.8	699
	9355_Zika_PF_18R	SEQ ID NO: 109 ttaggatccTATGGGGGAGGACTGGT	71	74.1	
19	9356_Zika_PF_19F	SEQ ID NO: 110 taactcGAGCCCAGAACCTTGGATC	73.3	75.5	711
	9357_Zika_PF_19R	SEQ ID NO: 111 ttaggatcCAGACCCCCAAGAAGGC	71.3	76.9	
20	9358_Zika_PF_20F	SEQ ID NO: 112 taactcgagCCCCTTTGGTCTTGTCT	71.7	75	706
	9359_Zika_PF_20R	SEQ ID NO: 113 ttaggatccAGGAAGGATGTATGCAGATG	71.9	73.9	
21	9360_Zika_PF_21F	SEQ ID NO: 114 taactcgagACATTTGCGCATATGATTTTG	70.4	75.7	709
	9361_Zika_PF_21R	SEQ ID NO: 115 ttaggatccAGGAAGGACACACAAGAGT	71.8	75	
22	9362_Zika_PF_22F	SEQ ID NO: 116 taactcgagACAGGCTGCACAGCTTT	70	79.1	581
	9363_Zika_PF_22R	SEQ ID NO: 117 ttaggatccTCTCTCATAGGGCACAGAC	74.8	81.1	

SEQUENCES

SEQ ID NO: 1
A typical form of protamine
PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

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Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 2
KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome

GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTGG
ATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACG
CGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGG
ATGGTCTTGGCAATTCTAGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTG
GGGAAAAAAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGA
AGGAGAAGAAGAGACGGGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGG
TCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACGATGCTGGGGAGGCCATATCTTTTCCAACCACATTG
GGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCT
GGATGAGGGGGTGGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCAT
CACAAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGT
CGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTC
GCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCT
GATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGG
GTTGATATTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTAC
AACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCT
GCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGC
TGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGTCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCG
GGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGT
TAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACC
CTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATG
AATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAAC
TCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGG
AGTCAAGAAGGAGCAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCT
CTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCA
CATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTG
CAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCA
CTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGG
GAGAAGAAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTGAAGCCACTGTGAGAGGTGCCA
AGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCTCAACTCATTGGGCAAGGGCAT
CCATCAAATTTTTGGAGCAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACAAATTCTCATTGGAACGTTGCTG
ATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGGGGGAGTGTTGATCTTCTTATCC
ACAGCCGTCTCTGCTGATGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGATGCGGTACAGGGGTGTTCGTCT
ATAACGACGTTGAAGCCTGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGATTGGCAGCAGCAGTCAAGCA
AGCCTGGGAAGATGGTATCTGCGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTGGAGATCAGTAGAAGGGGAG
CTCAACGCAATCCTGGAAGAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTC
CACAGAGATTGCCCGTGCCTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGCACTTCGTCAGAGCAGC
AAAGACAAATAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTC
TTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGAT
CCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGA
ATGACACATGGAGGCTGAAGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGAC
AGATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCT
ACAGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCA
CGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGATCGAGGAATGGTG
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SEQ ID NO: 3

KU497555.1 Zika virus isolate Brazil-ZKV2015, Brazil, complete genome

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SEQ ID NO: 4
KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome

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SEQ ID NO: 5
KU509998.1 Zika virus strain Haiti/1225/2014, Haiti, complete genome

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SEQ ID NO: 6

KU527068.1 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, complete genome

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SEQ ID NO: 7

KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome

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SEQ ID NO: 8
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SEQ ID NO: 9
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SEQ ID NO: 11

LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome

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SEQ ID NO: 12
AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome
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SEQ ID NO: 13
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GCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAG
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ATGGAGATAAGGCCCAGGAAAGAACCAGAAAGTAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGG
ATCACTTCTCCCTTGGAGTGCTTGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATC
ATAAGCACATCGATGGCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTT
GATGGGTGCCACCTTCGCGGAAATGAACACTGGAGGAGATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGA
CCAGCGTTGCTGGTATCTTTCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTT
TGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGATGGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAG
CGATGGTTGTTCCACGCACTGATAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTG
TGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAA
CTTACCATTTGTCATGGCCCTGGGACTAACCGCTGTGAGGCTGGTCGACCCCATCAACGTGGTGGGACTGCTGTTGCTCAC
AAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTC
GCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGA
GTGTGGACATGTACATTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCT
CGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAG
GTGGTCCTGATGACCATCTGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGAC
TGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTA
CAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGT
GGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATC
TGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCC
CGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCT
GGATTACCCAGCAGGAACTTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTC
GTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGC
CTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAA
ATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGA
AGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAAT
GTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCAC
TTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCAT
GACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAG
AGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAAC
GGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAAACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGT
TCCAGAAAACAAAACATCAAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGAC
CGTGTCATAGATTCCAGGAGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGT
CACACATGCCAGCGCTGCCCAGAGGAGGGGGCGCATAGGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGA
GGTGGGTGCGCAGAGACTGACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGA
TGGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAG
CAAAGGAAGACCTTTGTGGAACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAAT
AACCTACACAGATAGAAGATGGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTG
TGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGA
AGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACA
CATGACAGAGAGATTCCAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAA
GCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTT
TTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATG
TGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAG
CCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTAC
CGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAAC
CATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCA
GCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGT
ATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC

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CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGC
GTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACAC
AATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGT
CGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGA
ACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTA
CACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGG
CCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCC
CGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTG
GAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCA
CCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTA
TGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTG
ACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTT
GAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCA
GCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCG
AAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGA
AATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCAT
TGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGG
GCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAA
ACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAG
GAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGA
AAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATT
AGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGT
GGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGA
AACAAGGGGAATTTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGA
AGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGAGAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACA
AAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCAT
TGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGA
CATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGC
AACTCATTCGGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGAC
CAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATT
GATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAAACCCT
CAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCC
ATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGG
AGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCA
ATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAATGG
ATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACCACATGGAAGACAAGACCCCA
GTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGA
CTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTC
AGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTA
TAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACC
CCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGAT
CAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAG

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13 or 72. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13 or 72.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 14
isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 15
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
RDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 16
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXXXXXXXNR
AEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 17
ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMXXXXXGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 18
ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF

EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 19
ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 20
isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 21
isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 22
isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVE
FKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 23
MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 24

LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 25
isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 26
ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 27
AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 28
MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 29
MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH

AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 30
KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 31
isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 32
CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 33
Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 34
isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 35
KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 36
KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 37
isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 38
BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 39
MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 40
KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 41
Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 42
KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 43
isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 44
isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 45
PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 46
BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 47
H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 48
PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 49
KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 50
ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 51
103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 52
isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 53
KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 54
isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 55
isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 56
isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 57
isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 58
isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 59
isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNGTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 60
isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 61
ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 62
KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 63
isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 64
isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCPTQGEAYLD
KQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENR
AKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFK
DAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVE
VQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 65
isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 66
KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN

RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 67
isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 68
ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E.
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCPTQGEPSL
DKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 69
MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 70
5'-$(dIdC)_{13}$-3'
dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC

SEQ ID NO: 71
KLK peptide
KLKLLLLLKLK

SEQ ID NO: 72
ZIKV Sequence H/PF/2013 as sequenced
CAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTT
TCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTG
AGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCT
AGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAAAAGAGGCTA
TGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGAAGGAGAAGAAGAGAC
GAGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAG
TGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTT
ATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAA

CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCACAAAAAAGGTGAAGC
ACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGTCGCAAACCTGGTTGGAAT
CAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCC
ATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGC
ATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAAC
ATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTACAACAACAGTCAGCAACAT
GGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCGGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAA
GCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTG
GACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCC
AGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGACACAGGACATG
AAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACCCTGGGGGGTTTTGGAAG
CCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATGAATAACAAGCACTGGTT
GGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAACTCCACACTGGAACAACA
AAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAG
TTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCTCTGGCCACTTGAAATG
TCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCC
GGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAG
ATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAA
CTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGGGAGAAGAAGATCACCC
ACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTGAAGCCACTGTGAGAGGTGCCAAGAGAATGGCAGTCTT
GGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCTCAACTCATTGGGCAAGGGCATCCATCAAATTTTTGGAG
CAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACAAATTCTCATTGGAACGTTGCTGATGTGGTTGGGTCTGA
ACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGGGGGAGTGTTGATCTTCTTATCCACAGCTGTCTCTGCTGA
TGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGATGCGGTACAGGGGTGTTCGTCTATAACGACGTTGAAGCC
TGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGATTGGCAGCAGCAGTCAAGCAAGCCTGGGAAGATGGTA
TCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTGGAGATCAGTAGAAGGGGAGCTCAACGCAATCCTGGAA
GAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGC
CTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGTACTTCGTCAGAGCAGCAAAGACAAATAACAGCTT
TGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTCTTGTGGAGGATCATGGGT
TCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAG
CTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGAATGACACATGGAGGCTGA
AGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGACAGATGGAATAGAAGAGAG
TGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCTACAGGACCCAAATGAAAG
GGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGG
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68

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SEQ ID NO: 117
9363_Zika_PF_22R
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In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NOs: 14-69 or 72. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69 or 72.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Dr., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977 and Altschul et al., J. Mol. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were adapted to ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 9A and B). Briefly, the unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Zika Virus. As shown in Figure 10, non-infectious virus particle aggregates, HCP and other LMW impurities were removed by PS precipitation as shown by removal of aggregate shoulder in SEC-HPLC and no loss of infectious virus titer by PS treatment. Further optimization of the Zika purification protocol is provided below.

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):

- 5% CO_2, 35°C, MEM+2mM L-Glutamine + 10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01
- Virus Production without serum
- 5% CO_2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (accession number KJ776791.1, also SEQ ID NO: 13 herein) from French Polynesia. A sample was obtained from the European Virus Archive (EVAg; Ref-SKU: 001v-EVA1545). Based on this material, a research master seed bank (rMSB) was prepared on Vero cells as the cell substrate and the genomic sequence was checked by sequencing. Because the genomic sequence at the 5′and 3′flanking sequences of Zika virus strain H/PF/2013 was unknown, primers for sequencing were designed in those regions based on other Zika virus strains whereas the internal primers were designed from the published sequence (SEQ ID NOs: 74 to 117, see also Table A). The sequence obtained from the rMSB by use of these primers is provided by SEQ ID NO: 72. There was 100% overlap of the sequence with the published sequence of Zika Virus Strain H/PF/2013 (SEQ ID NO: 13). However, we sequenced additional regions 5′ (an additional 40 bp) and 3’ (an additional 160 bp) represented in SEQ ID NO: 72. In a preferred embodiment, the Zika virus of the invention comprises SEQ ID NO: 72. The genomic RNA is somewhat longer than the sequence according to SEQ ID NO: 72 (perhaps an additional 200 bp). Additionally, a Zika virus adapted to a host cell such as e.g. Vero cells may be expected to contain one or more mutations. For these reasons, the Zika virus of the current invention comprises the sequence of SEQ ID NO: 72 or, preferably, a sequence with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 72. Furthermore, because the viral genome is likely to contain even further flanking regions to SEQ ID NO: 72; in one embodiment, the Zika virus of the invention contains the sequence of SEQ ID NO: 72 and optionally further comprises extensions at the 5’ and/or 3’ ends of at least 10, at least 20, at least 30, at least 40, at least 50, at least

60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120 or at least 130 nucleotides. In a preferred embodiment, the Zika virus comprises at least the coding sequence for the entire polyprotein of Zika Virus Strain H/PF/2013 of the invention i.e. the amino acid sequence of SEQ ID NO: 73 or a polyprotein with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 73. Furthermore, the Zika virus comprises at least the coding sequence for the E-protein of Zika Virus Strain H/PF/2013 of the invention SEQ ID NO: 47 or an E-protein thereof with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 47.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in a standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and to reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles were infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200 mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significantly higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with the day 3 harvest, the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest.

Table 3: The calculated titers per plaque assay are summarized in the list below.

	Log 10 PFU/mL
sample day2	7.02
harvest day 3	6.66
harvest day 5	6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 15. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22°C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 11 for SEC-HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 20 for SEC-HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 21). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

Samples of individual 30x concentrated harvests days 2, 3, 5 and 7 were analysed before (Figure 11) and after PS (Figure 12) treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 4. The virus peak recovery by SEC-HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 4: ZikaV recovery by SEC-HPLC and plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x + PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x + PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles. Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus particles from residual contaminants as demonstrated for ChikV (Fig. 15D). The optimal volumes of

the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 5.

Table 5: Individual layers/volumes for a centrifugation in bottle.

Solution	**Volume (mL)**
PS treated harvest in 10% sucrose (L)	40
15% sucrose (J)	15
35% sucrose (I)	15
50% sucrose (H)	20
Total volume	90

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 14. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled rotor. (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers.) The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no brake/deceleration activated. In case a different centrifuge system with a different rotor is used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in

position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle (see Figure 14).

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL) and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 14. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51 mL in a PETG container. A volume of 1% formaldehyde (10,000 ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200 ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25ºC) for a minimum of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criterion for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution was monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152 µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. Briefly, a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 21.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gently mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 6).

Table 6: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	**JEV**	**Zika**
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2 µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565.

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 16. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 17) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6 nm and ~49 nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 18).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Table 7: Active ZikaV pfus were quantified by plaque assay throughout the process.

Sample	**Pfu/mL**
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$

Harvest day 5 (filtered)	$1.5x10^8$
Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD
Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2 mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly, the Benzonase treated samples did not yield any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell protein was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well was seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5% CO_2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicate. The target viruses (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were diluted to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were incubated at 35°C with 5% CO_2 for 1 hour. The cell culture medium was aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plates were rocked back and forth 5 times before incubating for 2 hours at 35°C with 5% CO_2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5% CO_2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 19 and 20, respectively). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Another neutralization assay was performed using the microneutralization assay as described by Larocca, et al. (2016, Nature doi:10.1038/nature18952). It was found that the inactivated Zika virus of the current invention had an MN50 (microneutralization) titer of 90 at 1 µg of inactivated purified virus.

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples

are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, http://dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on Vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV vaccine. The neutralization of both the Asian

(H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

The in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, supra.). Inactivated viruses are among the safest vaccines and especially preferred for deliver to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

A4. The vaccine of any one of A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of A1-A9, further comprising an adjuvant.

A11. The vaccine of A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

A15. The vaccine of any one of A1-A14, further comprising one or more pharmaceutically acceptable excipient.

A16. The vaccine of any one of A1-A15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 µg/mL, especially below 100 ng/mL.

A17. The vaccine of A16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

B1. A kit comprising a Zika virus vaccine of any one of A1-A15.

B2. The kit of B1, further comprising a second vaccine.

B3. The kit of B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of A1-A15 to a subject in need thereof.

C2. The method of C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of A1-A15 for the treatment and/or prevention of a Zika virus infection.

E2. The use of E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment or prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of A1-A15.

F2. The pharmaceutical composition of F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

G1. Use of an optimized sucrose gradient centrifugation for removal of protamine sulphate from purified infectious Zika virus particles.

G2. The use according to G1, wherein said optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three further layers of sucrose solutions with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G3. A process of purification of infectious Zika virus particles, comprising the steps of:

a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

G4. The process of G3, wherein said optimized sucrose density gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G5. The process of any one of G3 to G4, additionally comprising a further purification step of: (d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

G6. The process of any of G3 to G5, wherein the residual host cell DNA content of the Zika virus preparation (c) is less than 10 ng/mL and the residual host cell protein content of the final virus preparation (c) is less than 100 ng/mL.

G7. The process of any of G3 to G6, wherein said crude harvest (a) comprising Zika virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

G8. The process of G7, wherein the one or more pre-purification step(s) comprises

a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or
b) digestion of host cell genomic DNA by enzymatic treatment; and/or
c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

G9. The process of any one of G3 to G8, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

G10. The process of any one of G3 to G9, wherein the enrichment of infectious Zika virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

G11. The process of any one of G7 to G10, wherein the one or more pre-purification step(s) prior to step (b) of any of G8 to G11 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

G12. The process of any one of G3 to G11, wherein the residual impurity of the Zika virus preparation (c) is less than 10%.

G13. The process of any one of G3 to G12, wherein the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

G14. The process of G13, wherein said cell line is a Vero cell line.

G15. The process of any one of G3 to G14, wherein said infectious Zika virus particle is an infectious virus particle that is a live virus, a live attenuated virus, a chimeric virus, a modified live virus, or a recombinant live virus.

G16. The process of any one of G3 to G15, wherein said Zika virus is preferably a strain of the Asian lineage.

G17. The process of any one of G3 to G16, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

G18. Use of the process according to any one of G3 to G17 for manufacturing a composition for immunization against a virus infection.

G19. The use according to G18, wherein said virus infection is an infection caused by a Zika virus.

Q1. A process of purification of infectious Zika virus particles, comprising the steps of:
(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

Q2. The process of Q2, wherein the virus particles are from Zika virus.

Q3. The process of Q1 or Q2, additionally comprising the step of:

(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

Q4. The process of any of Q1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

Q5. The process of any of Q1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

Q6. The process of Q5, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

Q7. The process of any one of Q1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

Q8. The process of any one of Q1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

Q9. The process of any one of Q5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of Q5 to 8 is performed using a filter having a pore size equal to or less than 1 µm, preferably 0.2 µm.

Q10. The process of any one of Q1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

Q11. The process of any one of Q1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

Q12. The process of Q11, wherein said cell line is a Vero cell line.

Q13. The process of any one of Q1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

Q14. The process of any one of Q1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

Q15. The process of any one of Q1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

Q16. Use of the process according to any one of Q1 to 15 for manufacturing a composition for immunization against a virus infection.

Q17. The use according to Q16, wherein the composition for immunization against a virus infection is an infection caused by Zika virus.

Q18. A composition comprising the virus particles obtainable or obtained by the process of any one of Q1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

Q19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

Q20. The Zika virus vaccine of Q19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

Q21. The vaccine of Q19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

Q22. The vaccine of any one of Q19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69 or 72, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 or 72 and able to pack a virulent Zika virus.

Q23. The vaccine of any one of Q19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

Q24. The vaccine of Q23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

Q25. The vaccine of Q24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

Q26. The vaccine of any one of Q19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

Q27. The vaccine of Q26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

Q28. The vaccine of Q27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

Q29. The vaccine of Q28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

Q30. The vaccine of any one of Q27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

Q31. The vaccine of any one of Q19 to 30, further comprising an adjuvant.

Q32. The vaccine of Q31, wherein the adjuvant is an aluminum salt adjuvant.

Q33. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

Q34. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

Q35. The vaccine of any one of Q19 to 34, further comprising one or more pharmaceutically acceptable excipient.

R1. Use of protamine, preferably a protamine salt, to separate infectious and non-infectious Zika virus particles, host cell proteins and/or undefined low molecular weight materials.

R2. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b), wherein the enrichment of infectious virus particles in the virus preparation (b) relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R3. The use of R1 or the process of R2, wherein the virus particles are from Zika virus.

R4. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by one or more size exclusion methods such as (i) a sucrose density gradient centrifugation, (ii) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles, and/or (iii) size exclusion chromatography to obtain a virus preparation (c) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (c) is less than 100 ng/mL and the residual host cell protein and the residual aggregates of infectious virus particles of the final virus preparation (c) is less than 1 µg/mL.

R5. The process of R4, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

R6. The process of any of R2 to 5, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

R7. The process of R6, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

R8. The process of any one of R2 to 7, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.

R9. The process of any one of R2 to 8, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R10. The process of any one of R6 to 9, wherein the one or more pre-purification step(s) prior to step (b) of any of R6 to 9 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

R11. The process of any one of R2 to 10, wherein the residual impurity of the virus preparation (c) is less than 10%.

R12. The process of any one of R2 to 11, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

R13. The process of R12, wherein said cell line is a Vero cell line.

R14. The process of any one of R2 to 13, wherein the Zika virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

R15. The process of any one of R2 to 14, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

R16. The process of any one of R2 to 15, wherein said process resulting in final virus preparation (c) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

R17. Use of the process according to any one of R1 to 16 for manufacturing a composition for immunization against a virus infection.

R18. The use according to R17, wherein the composition for immunization against a virus infection is an infection caused by a Zika virus.

R19. A composition comprising the virus particles obtainable or obtained by the process of any one of R2 to 16 for treating and/or preventing an infection.

5

CLAIMS

What is claimed is:

1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein said Zika virus vaccine is able to confer seroprotection on at least 70% of subjects that are administered the Zika virus vaccine.

2. The Zika virus vaccine of claim 1, wherein the Zika virus particle is able to confer seroprotection on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects that are administered the Zika virus vaccine, preferably on at least 80% of subjects.

3. The vaccine of claim 1 or 2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

4. The vaccine of any one of claims 1 to 3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

5. The vaccine of any one of claims 1 to 4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

6. The vaccine of claim 5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

7. The vaccine of claim 6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

8. The vaccine of claim 7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

9. The vaccine of any one of claims 5 to 8, wherein the chemical activation is performed at about +4°C or about +22°C.

10. The vaccine of any one of claims 1 to 9, further comprising an adjuvant.

11. The vaccine of claim 10, wherein the adjuvant is an aluminum salt adjuvant.

12. The vaccine of claim 11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

13. The vaccine of any one of claims 10 to 12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

14. The vaccine of claim 13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

15. The vaccine of any one of claims 1 to 14, further comprising one or more pharmaceutically acceptable excipients.

16. The vaccine of any one of claims 1 to 15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 µg/mL, especially below 100 ng/mL.

17. The vaccine of claim 16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

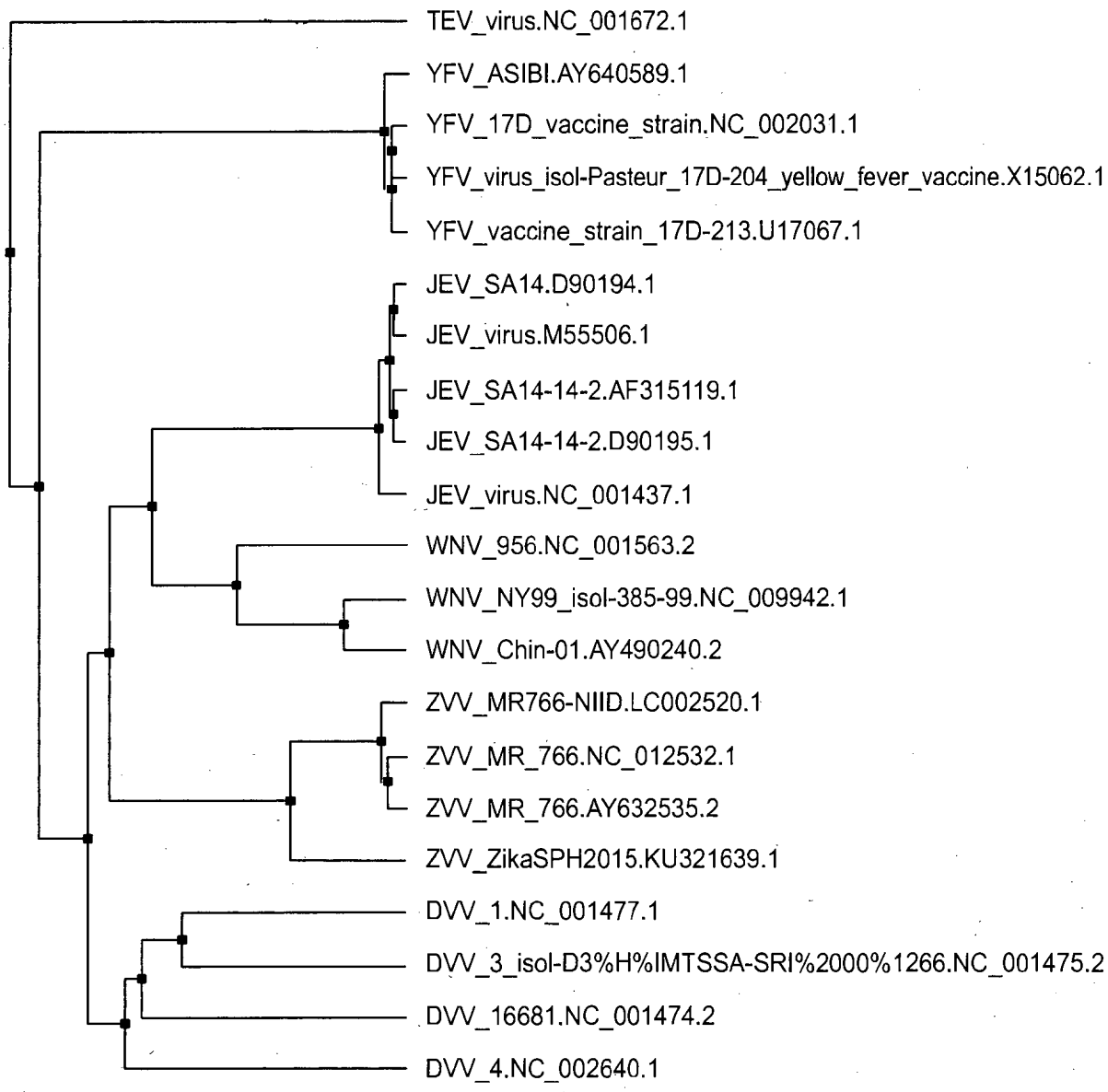

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Figure 1

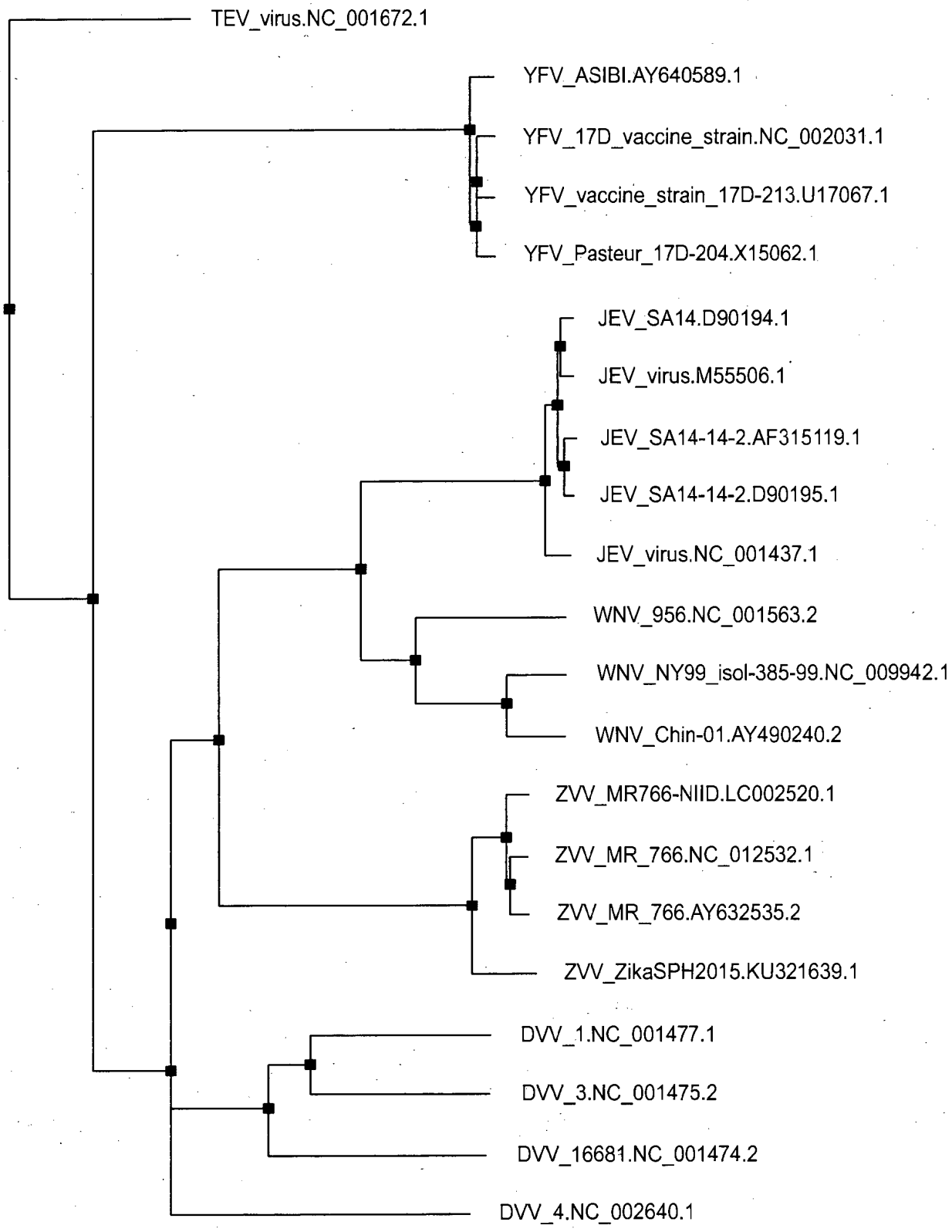

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Figure 2

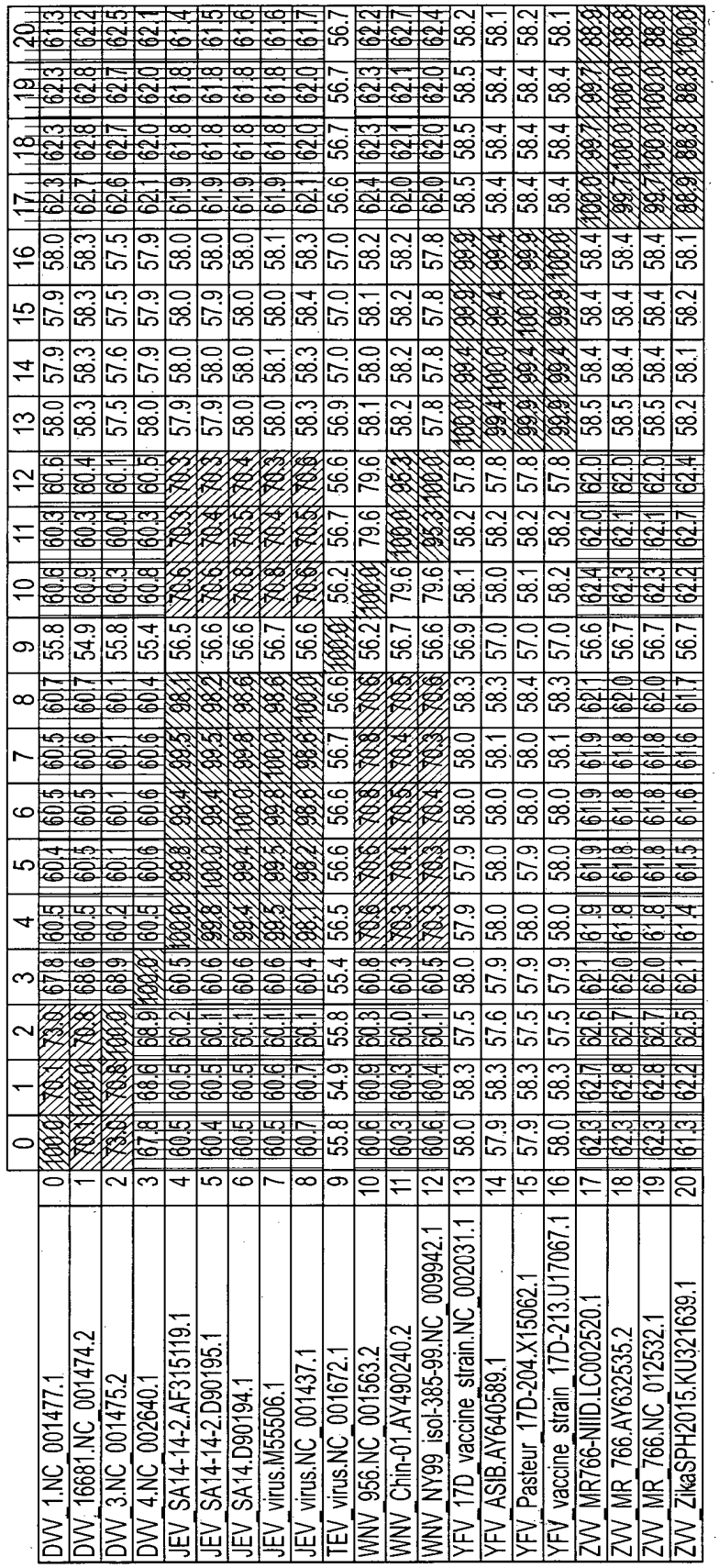

% Identity and higher
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Figure 3

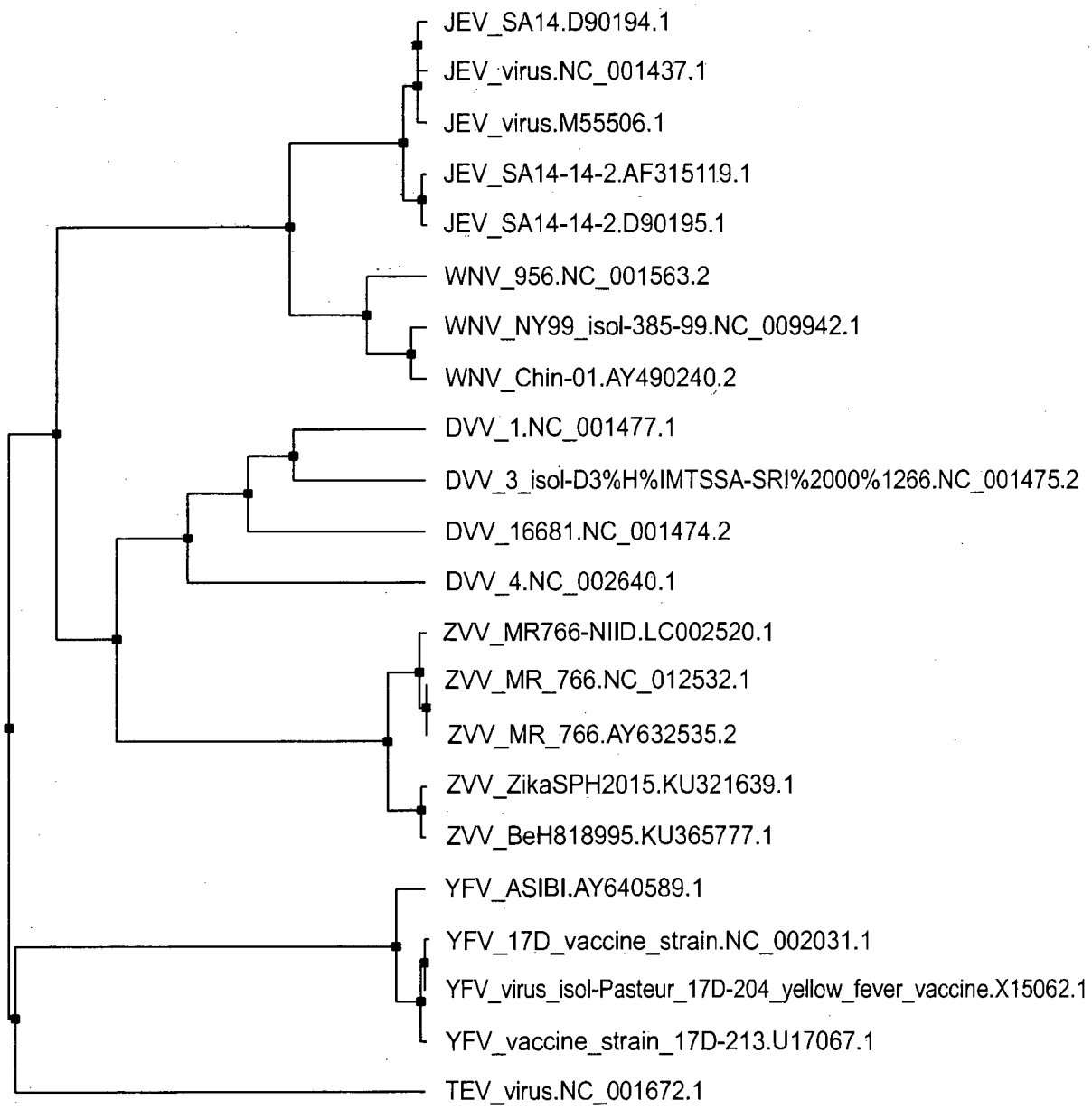

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Figure 4

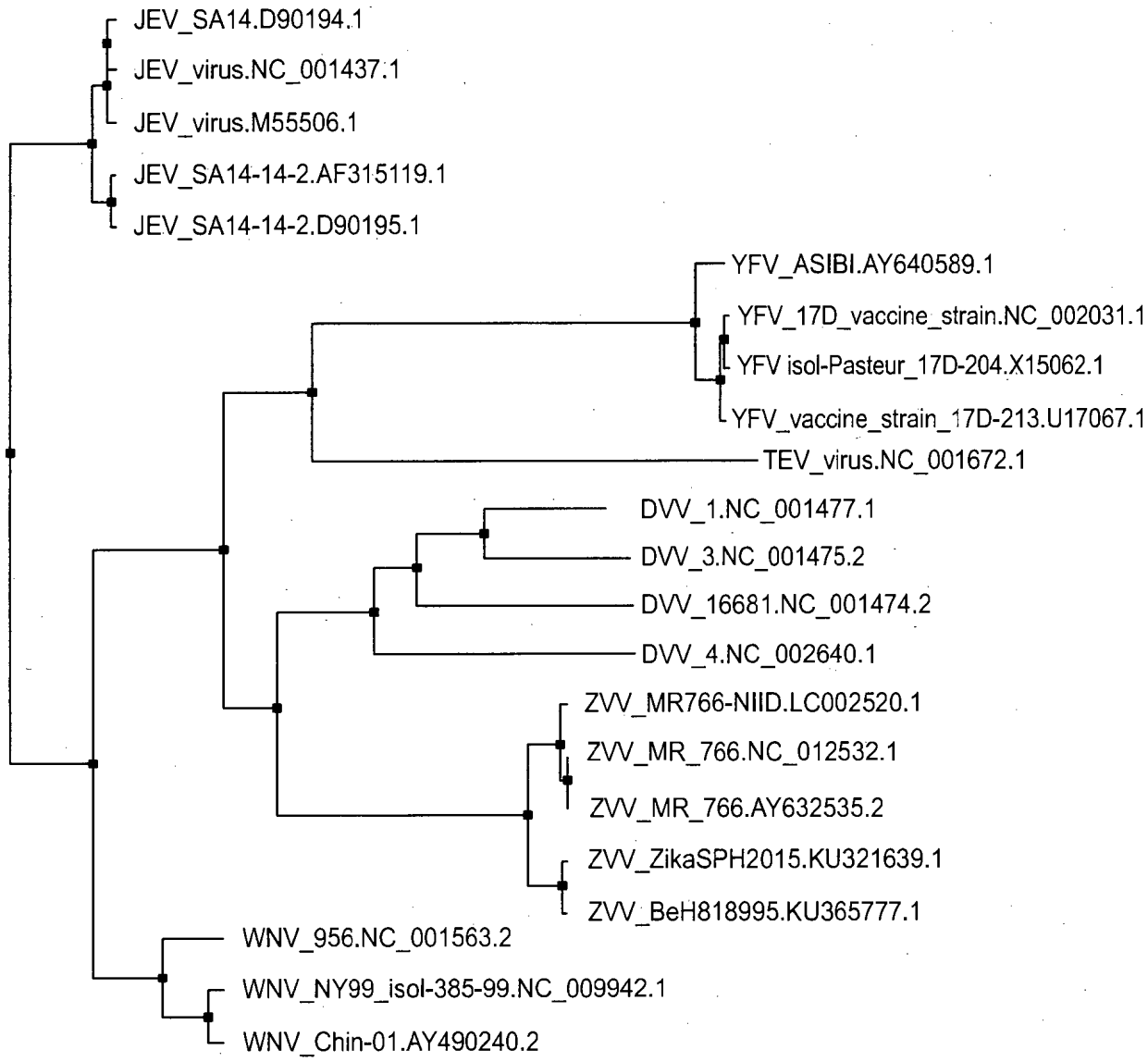

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WNV_Chin-01.AY490240.2

Figure 5

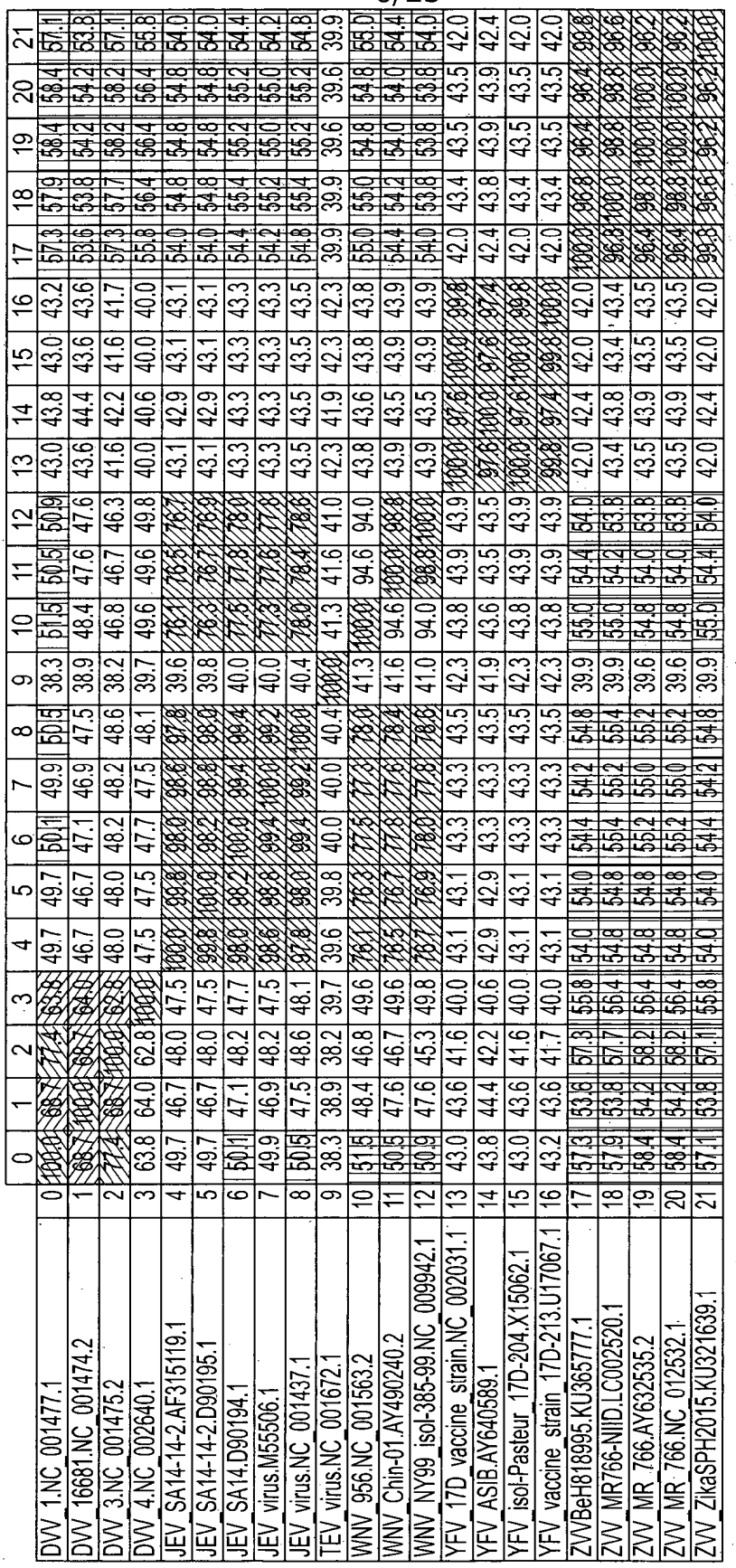

50 % Identity and higher
60 % Identity and higher
70 % Identity and higher

Figure 6

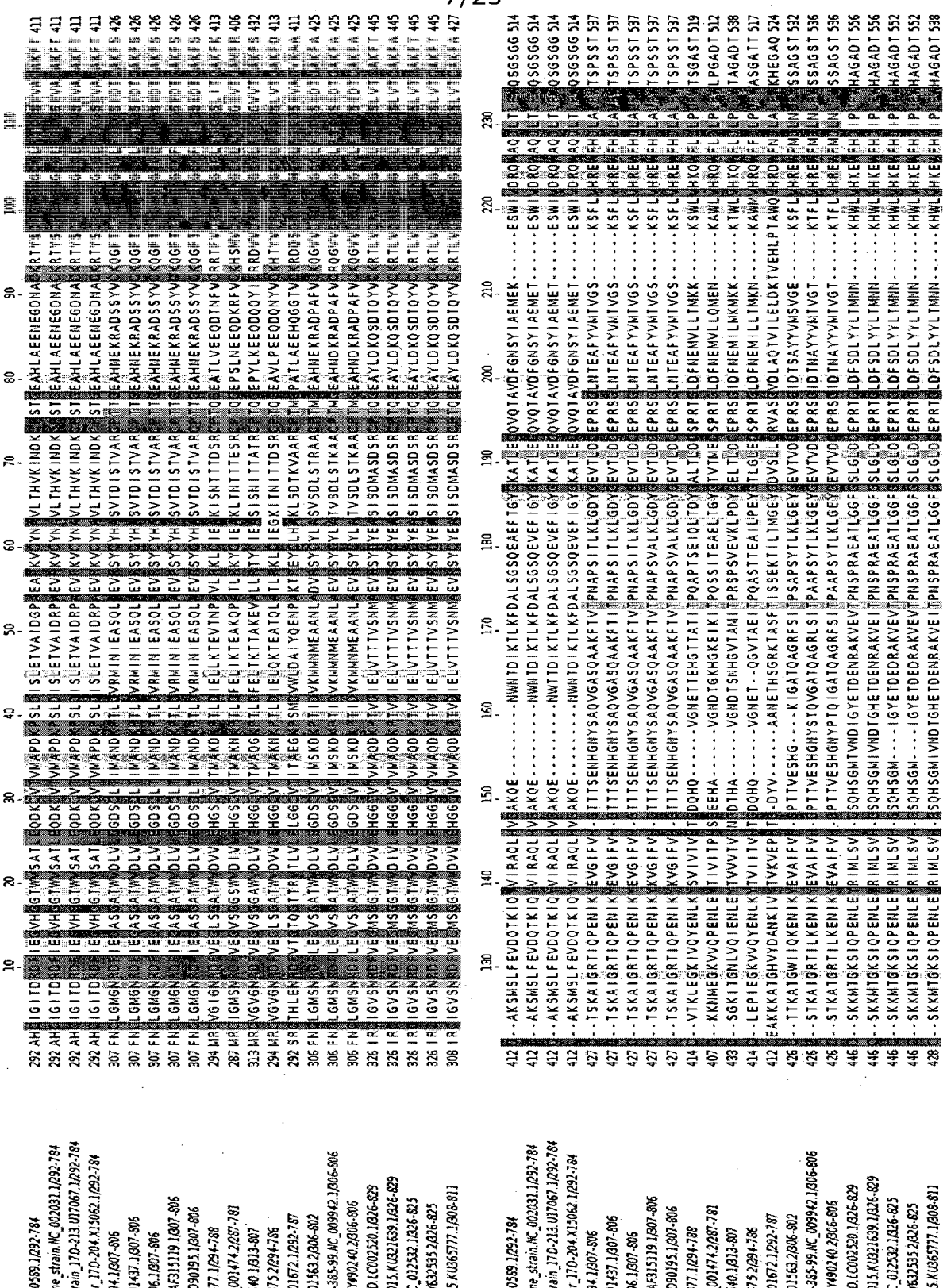


Figure 7 (Part 1 of 3)

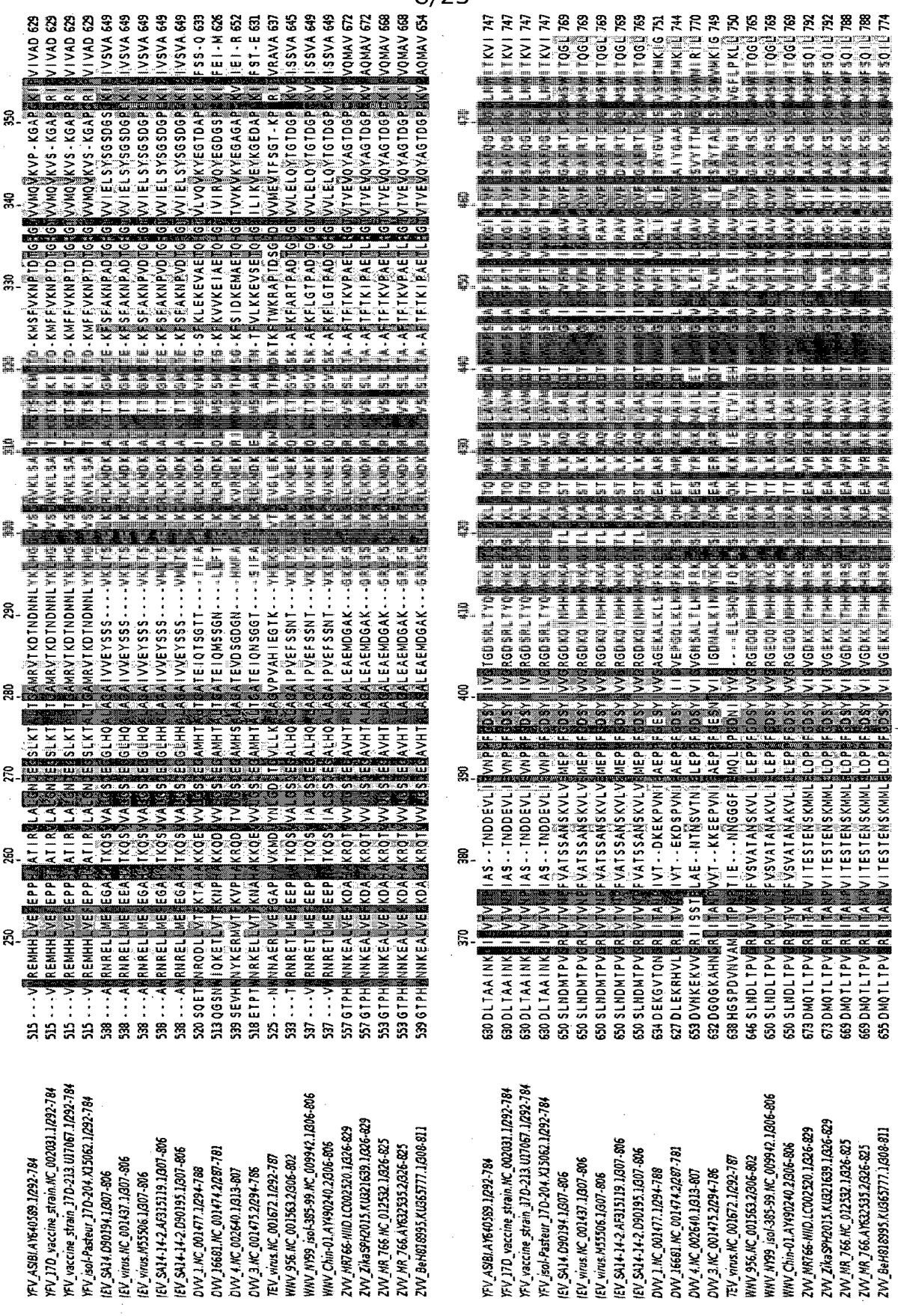


Figure 7 (Part 2 of 3)

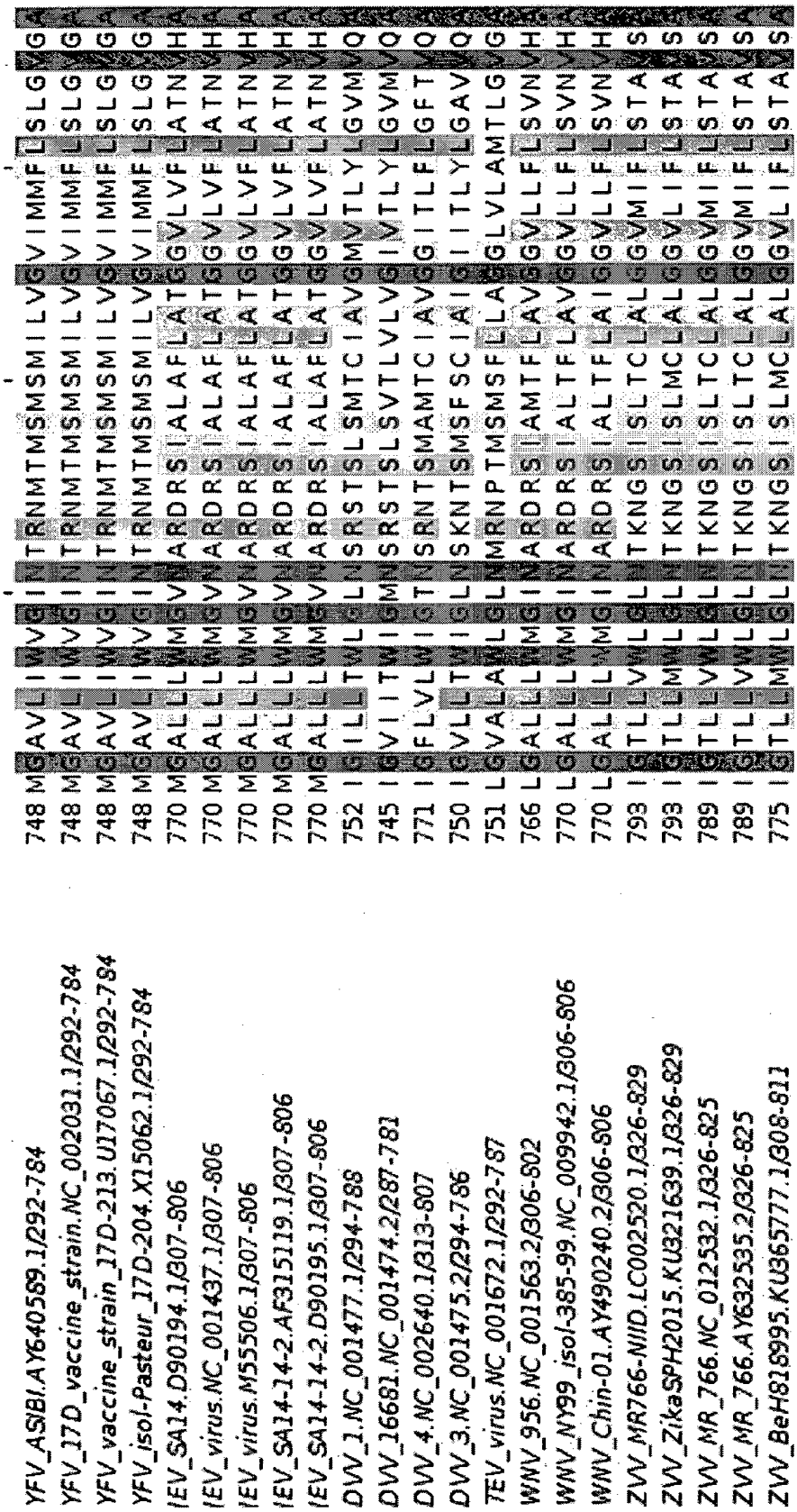


Figure 7 (Part 3 of 3)

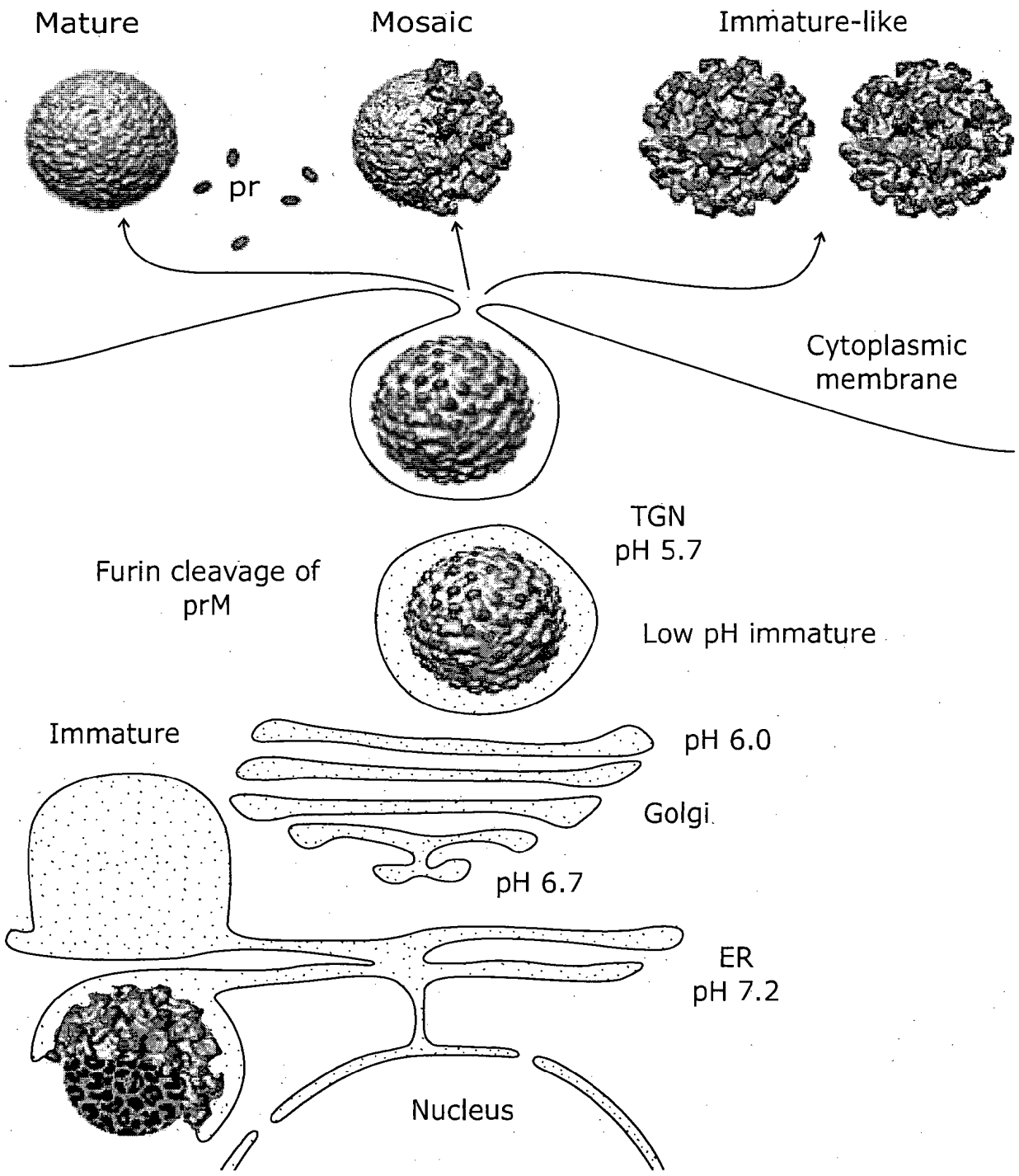

Mature
Mosaic
Immature-like
pr
Cytoplasmic membrane
TGN
pH 5.7
Furin cleavage of prM
Low pH immature
Immature
pH 6.0
Golgi
pH 6.7
ER
pH 7.2
Nucleus

Figure 8

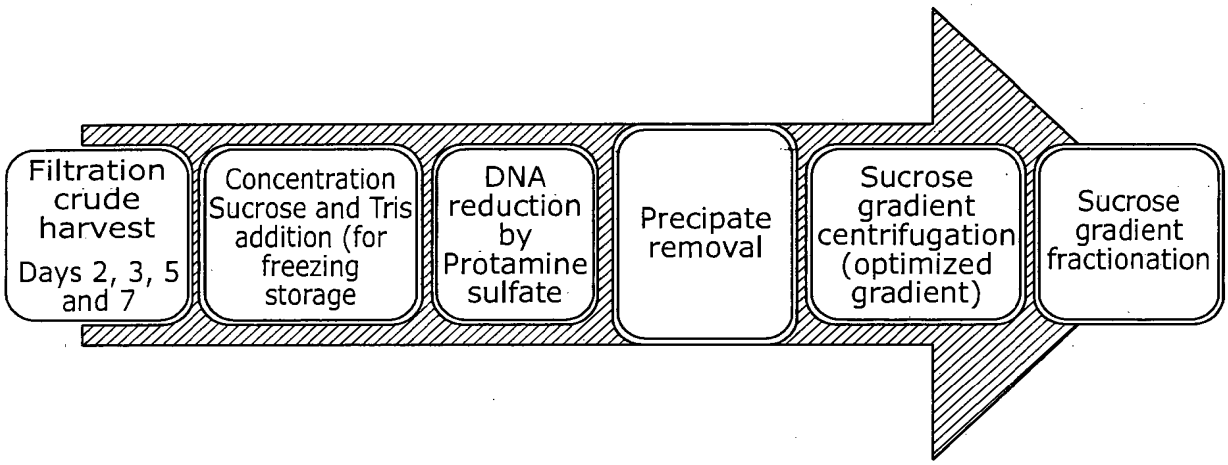


Figure 9A

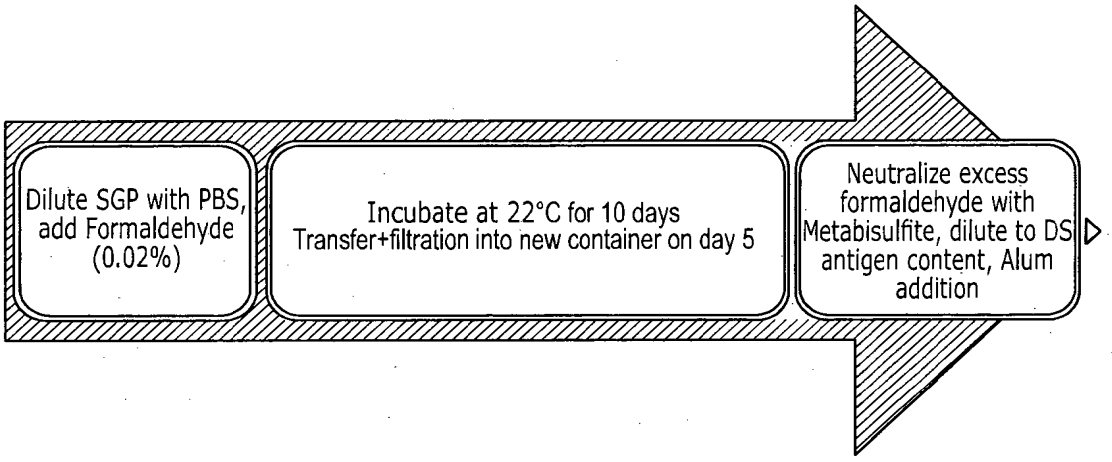


Figure 9B

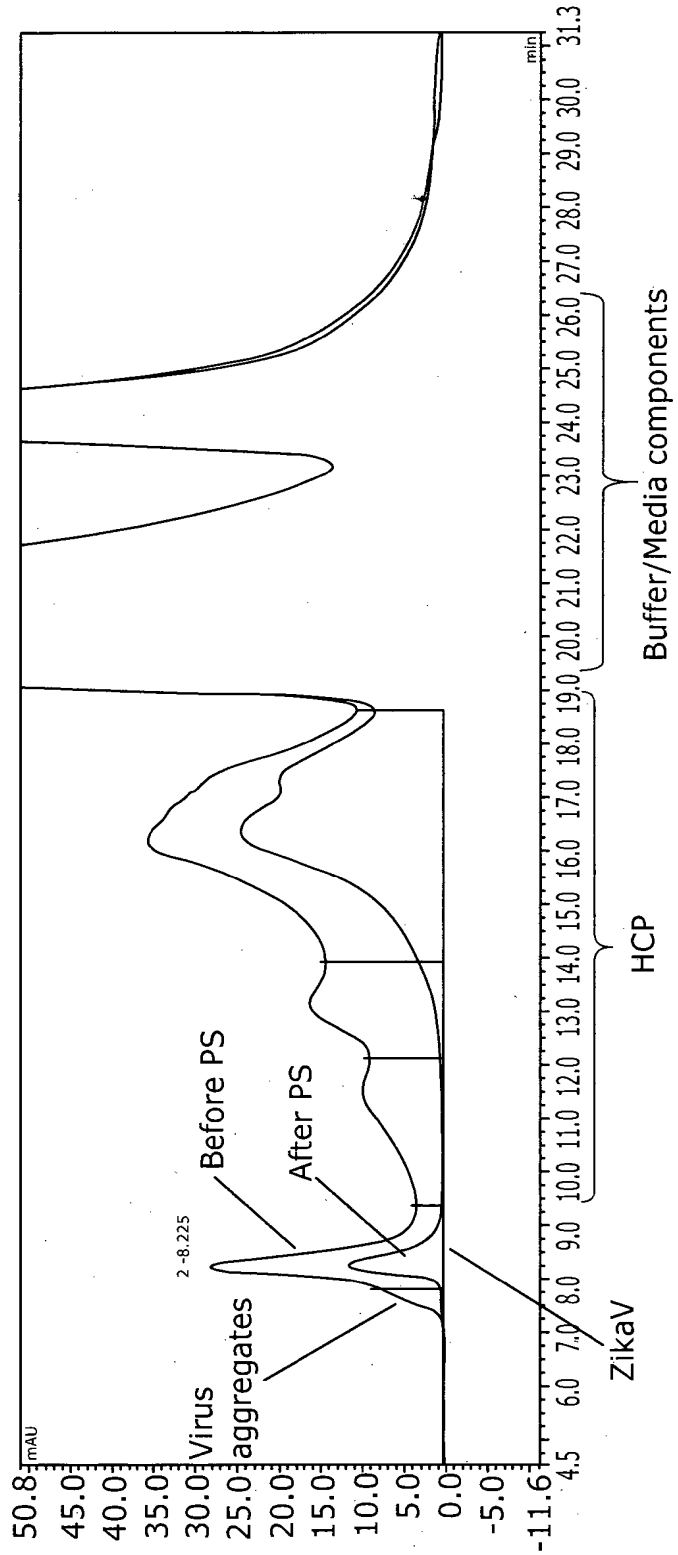
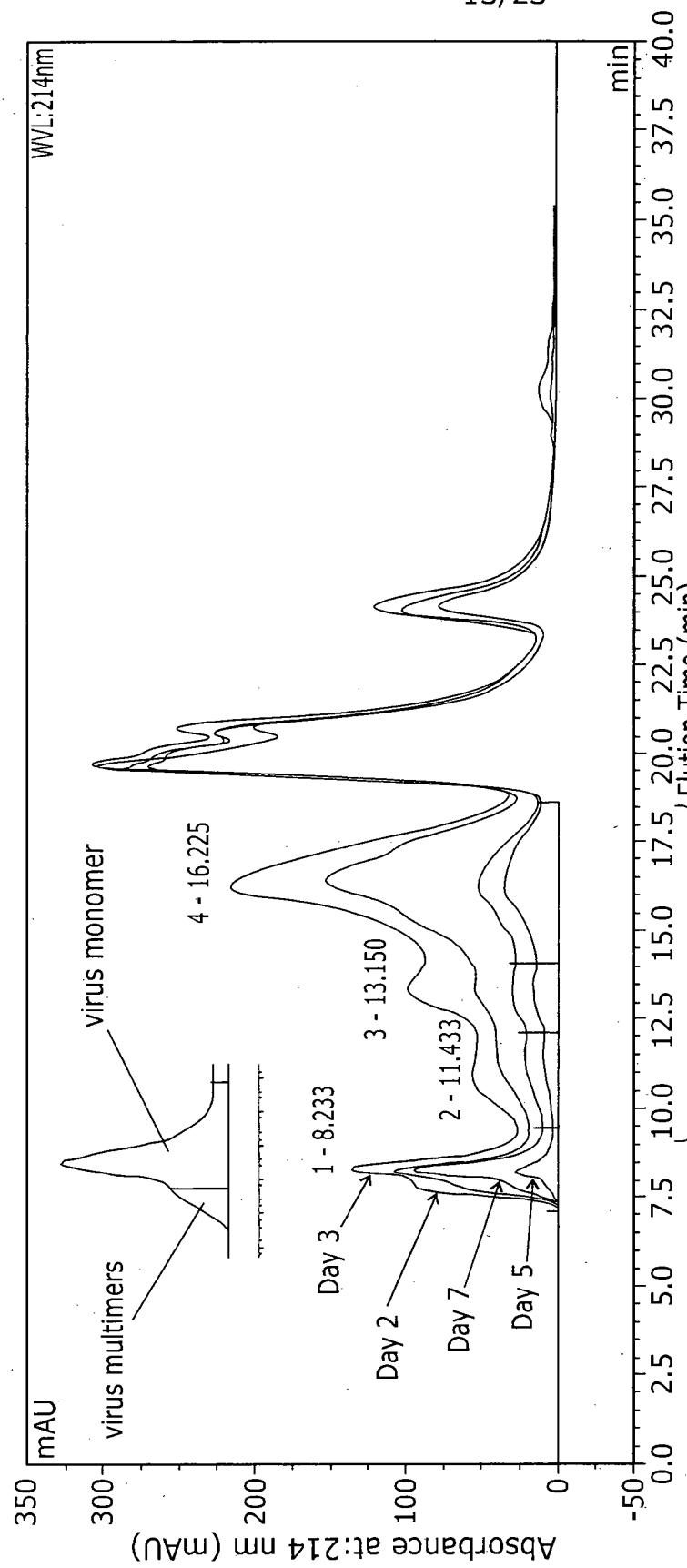

mAU
min
Virus aggregates
Before PS
After PS
ZikaV
HCP
Buffer/Media components
2 -8.225

Figure 10

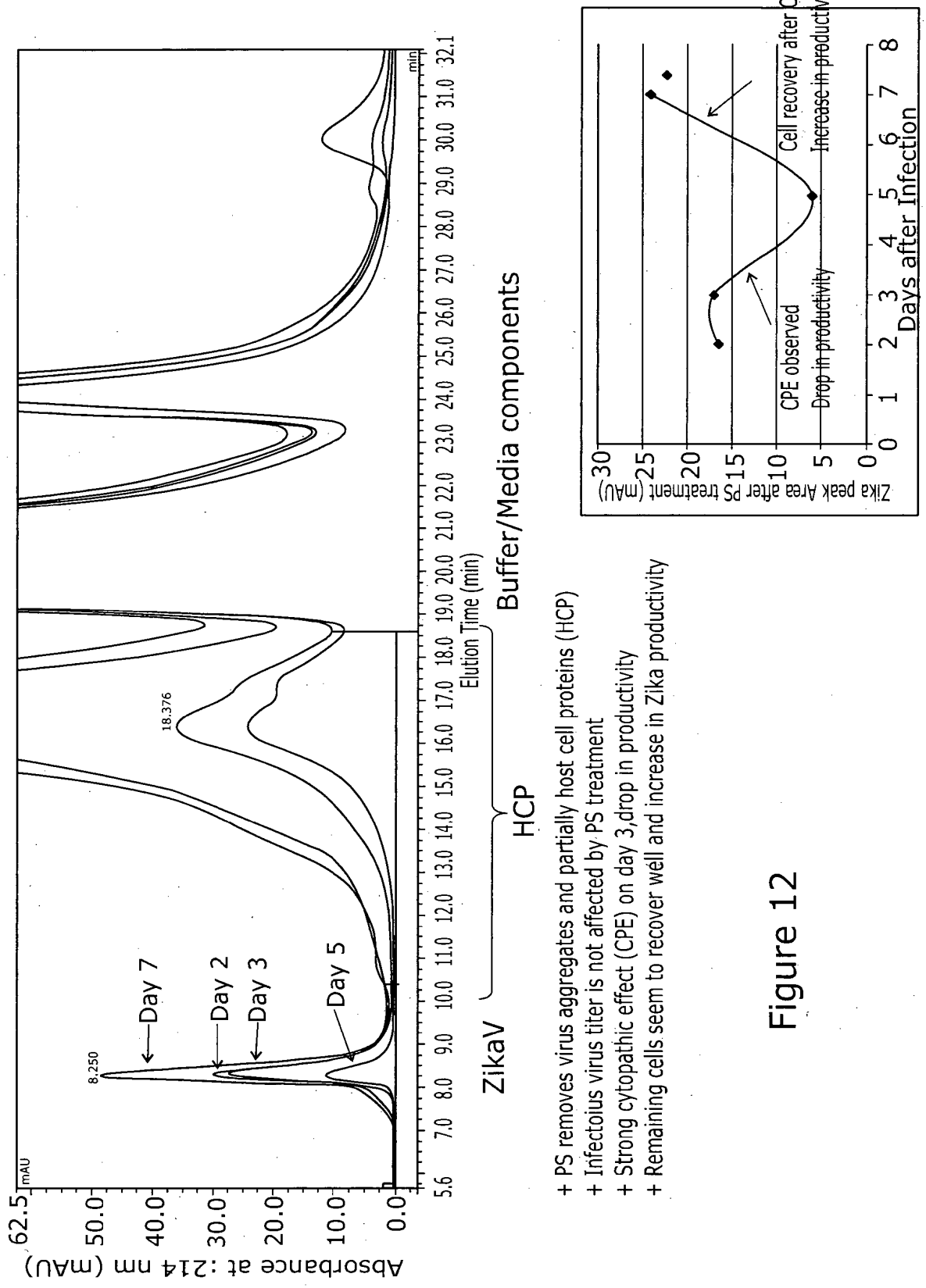


+ Similar virus concentration in harvests Day 2, Day3, Day 7; lower virus concentration in harvest Day 5

+ Day 2 and Day 3 harvests contained more HCP than Days 5/7 (correlates with observed CPE)

+ Minor amount of virus multimers observed

Figure 11



+ PS removes virus aggregates and partially host cell proteins (HCP)
+ Infectoius virus titer is not affected by PS treatment
+ Strong cytopathic effect (CPE) on day 3,drop in productivity
+ Remaining cells seem to recover well and increase in Zika productivity

Figure 12

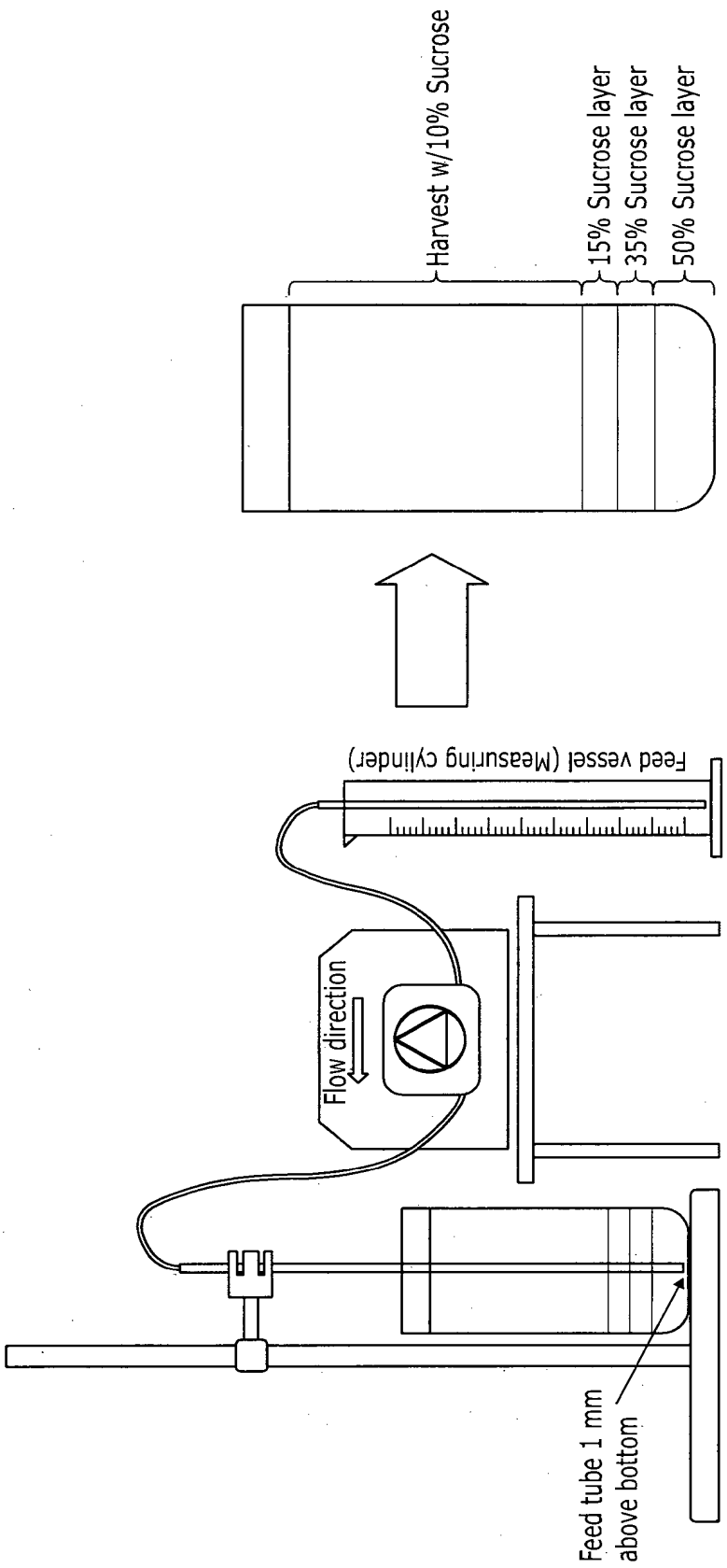
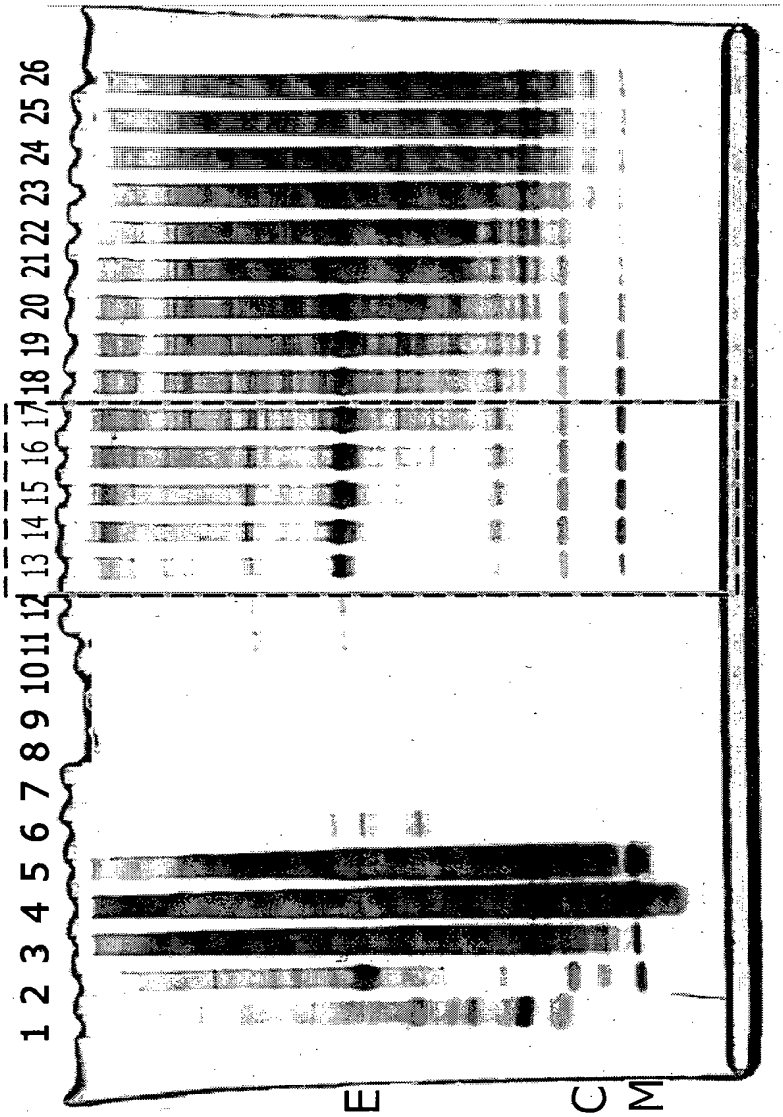

Harvest w/10% Sucrose
15% Sucrose layer
35% Sucrose layer
50% Sucrose layer
Feed vessel (Measuring cylinder)
Flow direction
Feed tube 1 mm above bottom

Figure 13

Lane	Sample
1	Molecular weight Marker
2	JEV Sucrose Gradient Pool (Reference)
3	Zika Harvest pool (day 2,3,5,7)
4	Zika Harvest pool (day 2,3,5,7)concentrated 300x
5	Zika Harvest pool (day 2,3,5,7)concentrated 300x + Protamine sulfate
6	Ultrafiltration Permeate
7	SGC Bottle 1, Fraction 4
8	SGC Bottle 1, Fraction 5
9	SGC Bottle 1, Fraction 6
10	SGC Bottle 1, Fraction 7
11	SGC Bottle 1, Fraction 8
12	SGC Bottle 1, Fraction 9
13	SGC Bottle 1, Fraction 10
14	SGC Bottle 1, Fraction 11
15	SGC Bottle 1, Fraction 12
16	SGC Bottle 1, Fraction 13
17	SGC Bottle 1, Fraction 14
18	SGC Bottle 1, Fraction 15
19	SGC Bottle 1, Fraction 16
20	SGC Bottle 1, Fraction 17
21	SGC Bottle 1, Fraction 18
22	SGC Bottle 1, Fraction 19
23	SGC Bottle 1, Fraction 20
24	SGC Bottle 1, Fraction 21
25	SGC Bottle 1, Fraction 22
26	SGC Bottle 1, Fraction 23



Pooled fractions F10-F14
(10mL total volume pere bottle)

Figure 14

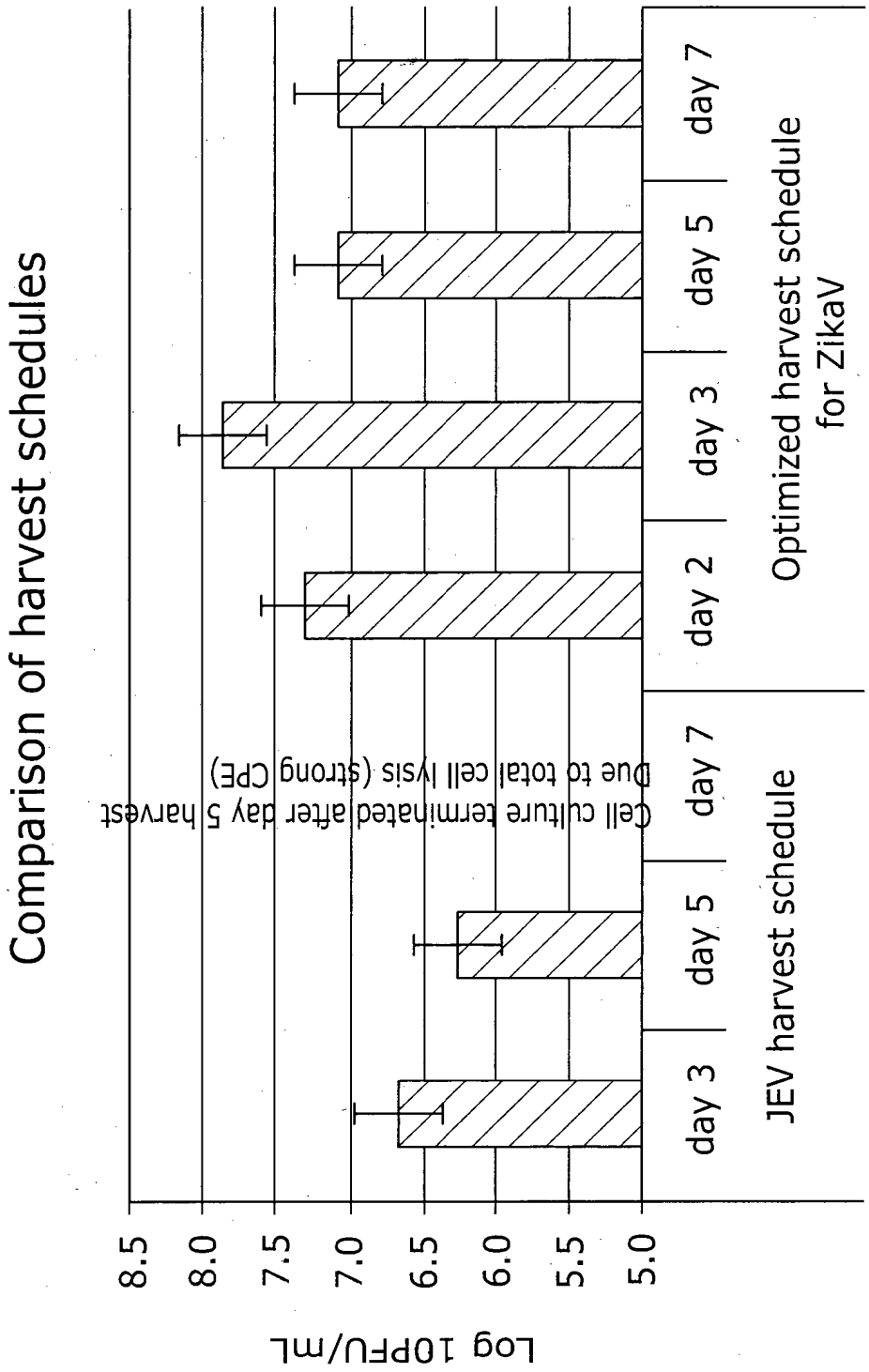

Comparison of harvest schedules
Log 10PFU/mL
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
day 3
day 5
day 7
JEV harvest schedule
day 2
day 3
day 5
day 7
Optimized harvest schedule for ZikaV
Cell culture terminated after day 5 harvest
Due to total cell lysis (strong CPE)

Figure 15

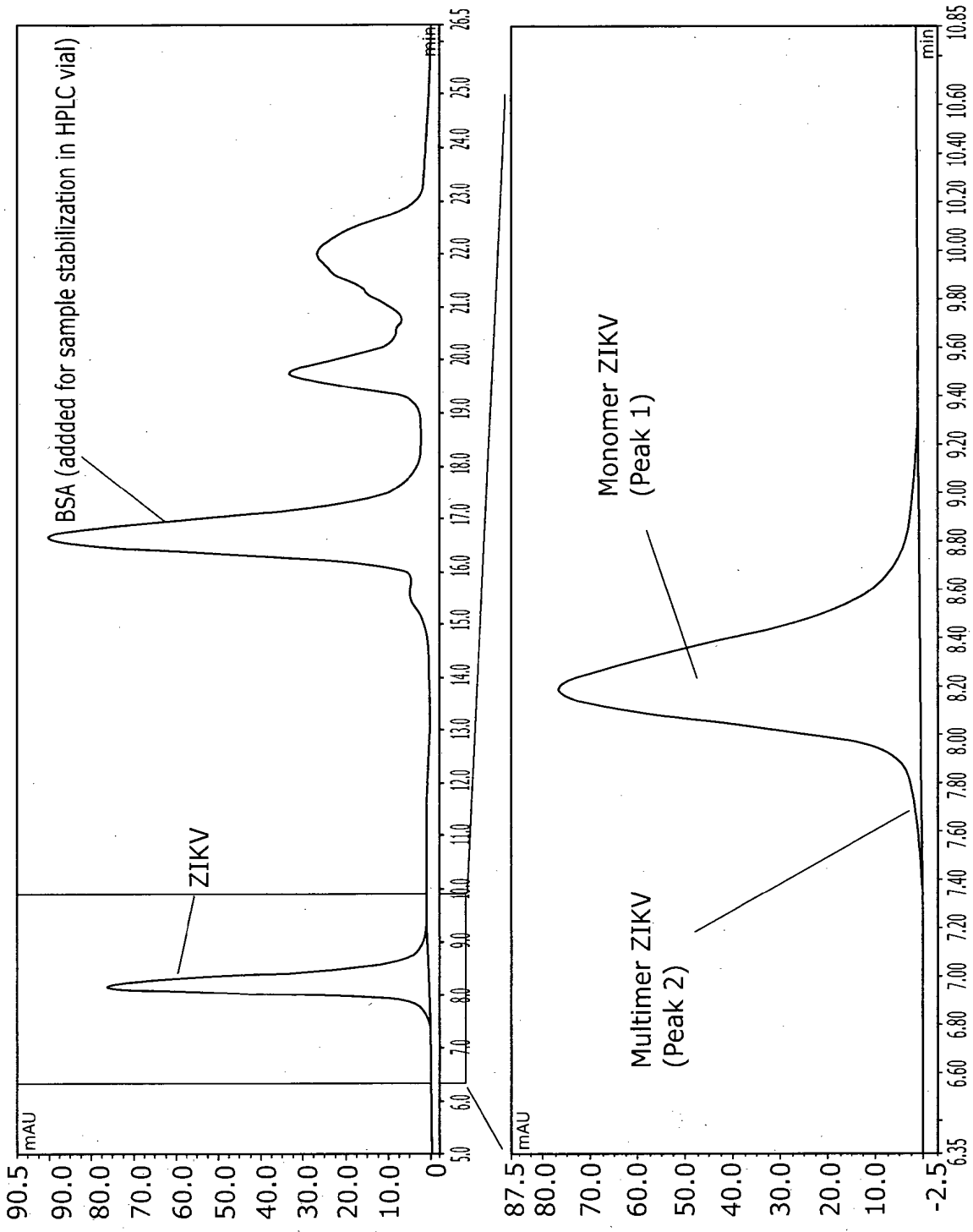

BSA (addded for sample stabilization in HPLC vial)
ZIKV
mAU
min
Monomer ZIKV
(Peak 1)
Multimer ZIKV
(Peak 2)
mAU
min

Figure 16

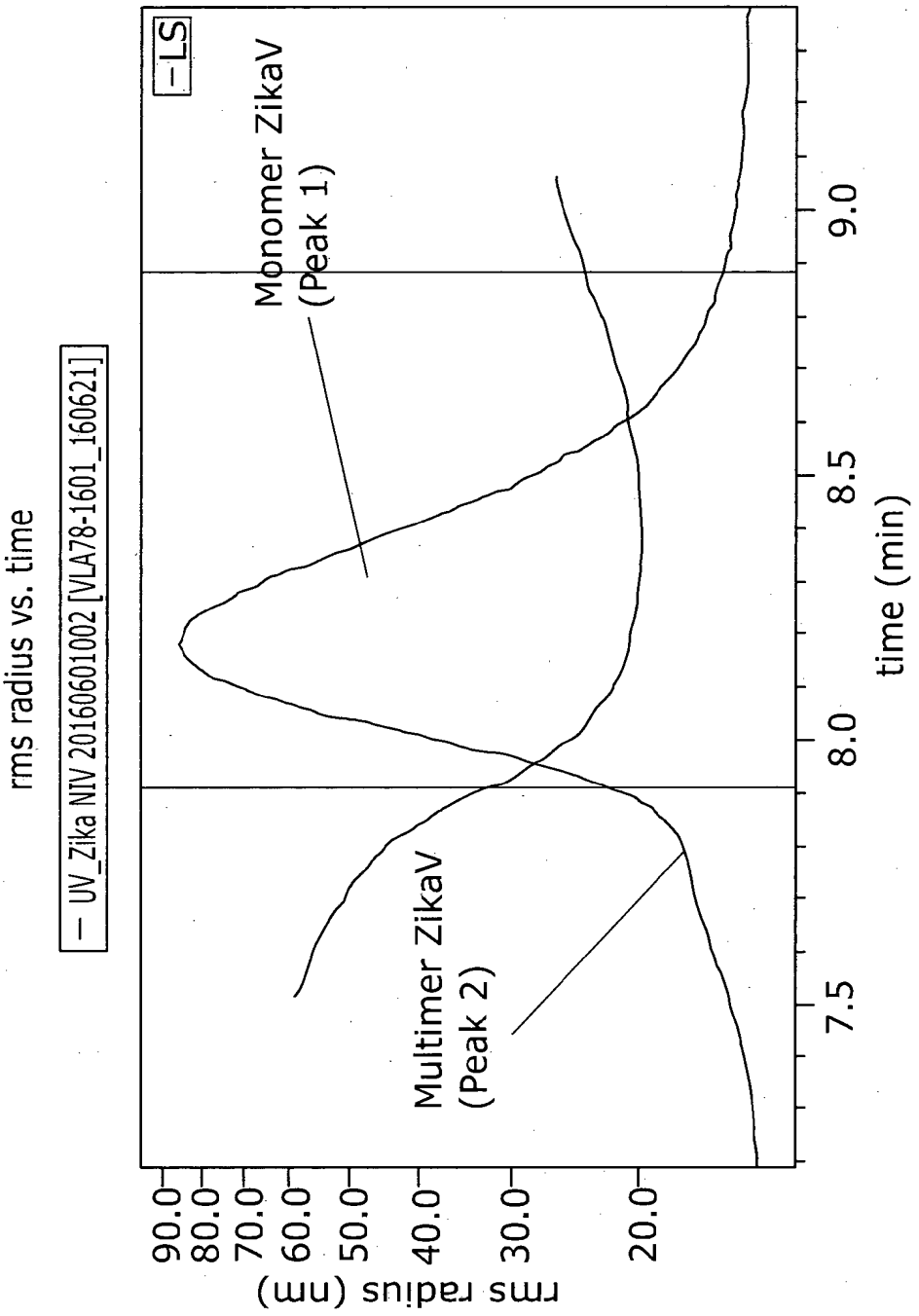

rms radius vs. time
– UV_Zika NIV 20160601002 [VLA78-1601_160621]
-LS
Monomer ZikaV
(Peak 1)
Multimer ZikaV
(Peak 2)
rms radius (nm)
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
7.5
8.0
8.5
9.0
time (min)

Figure 17

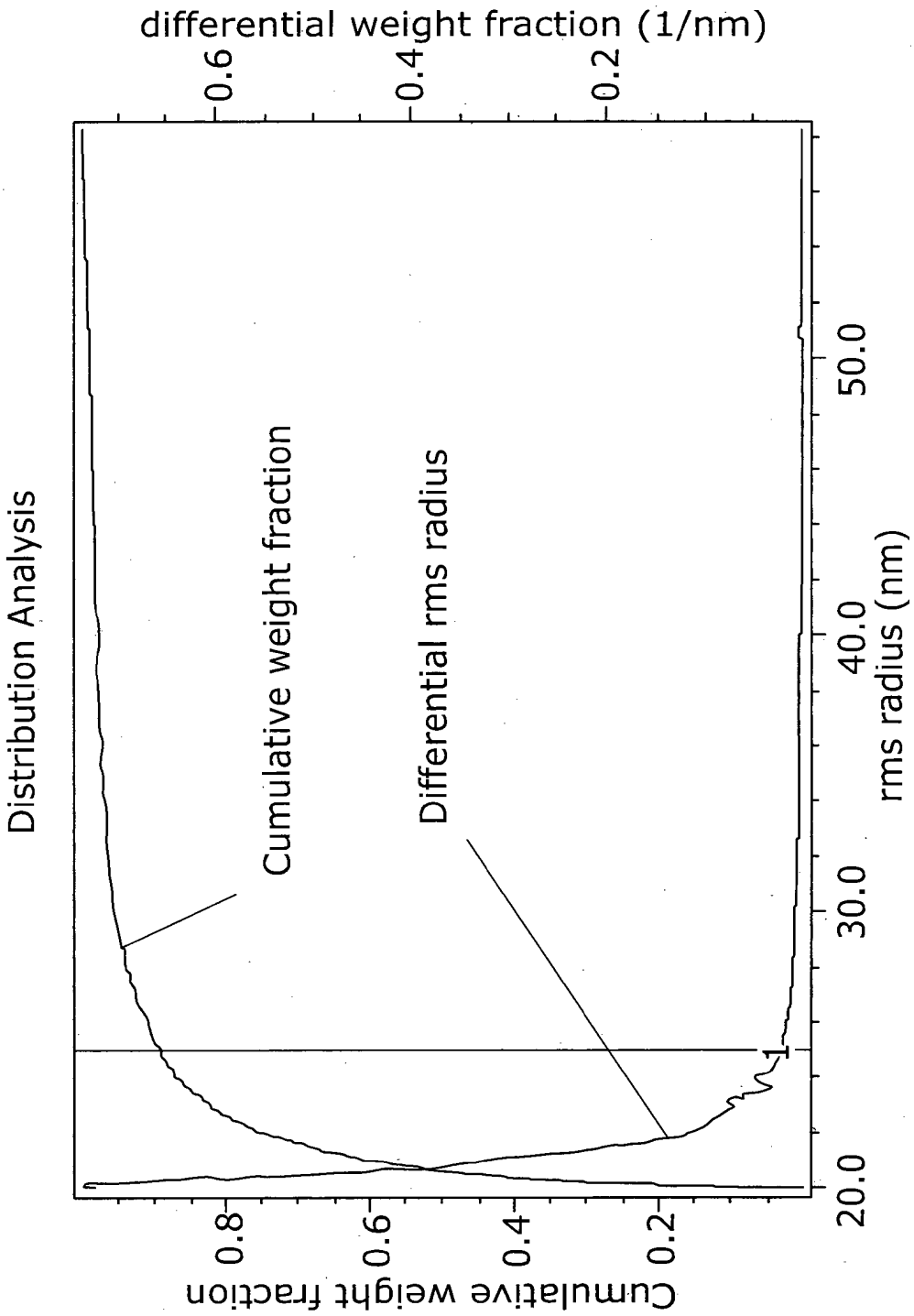
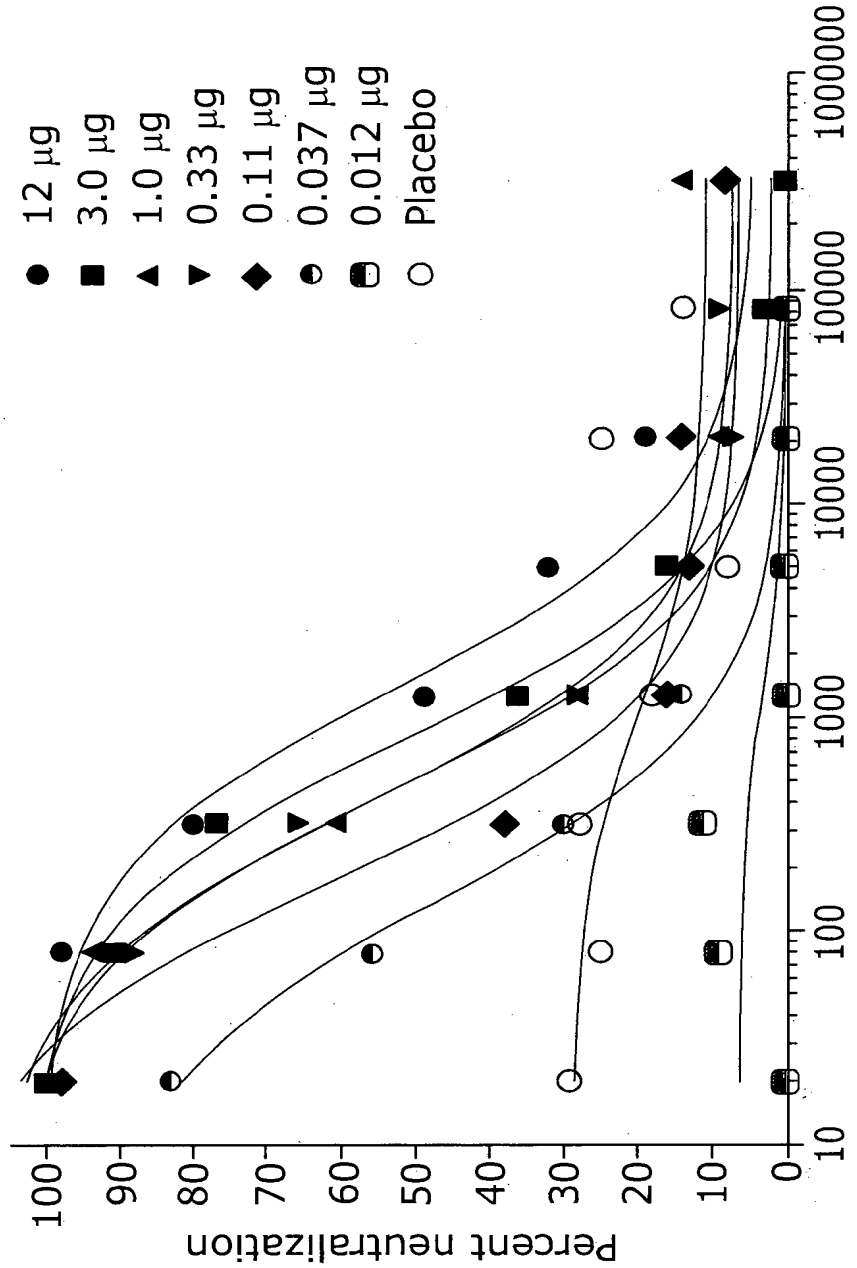
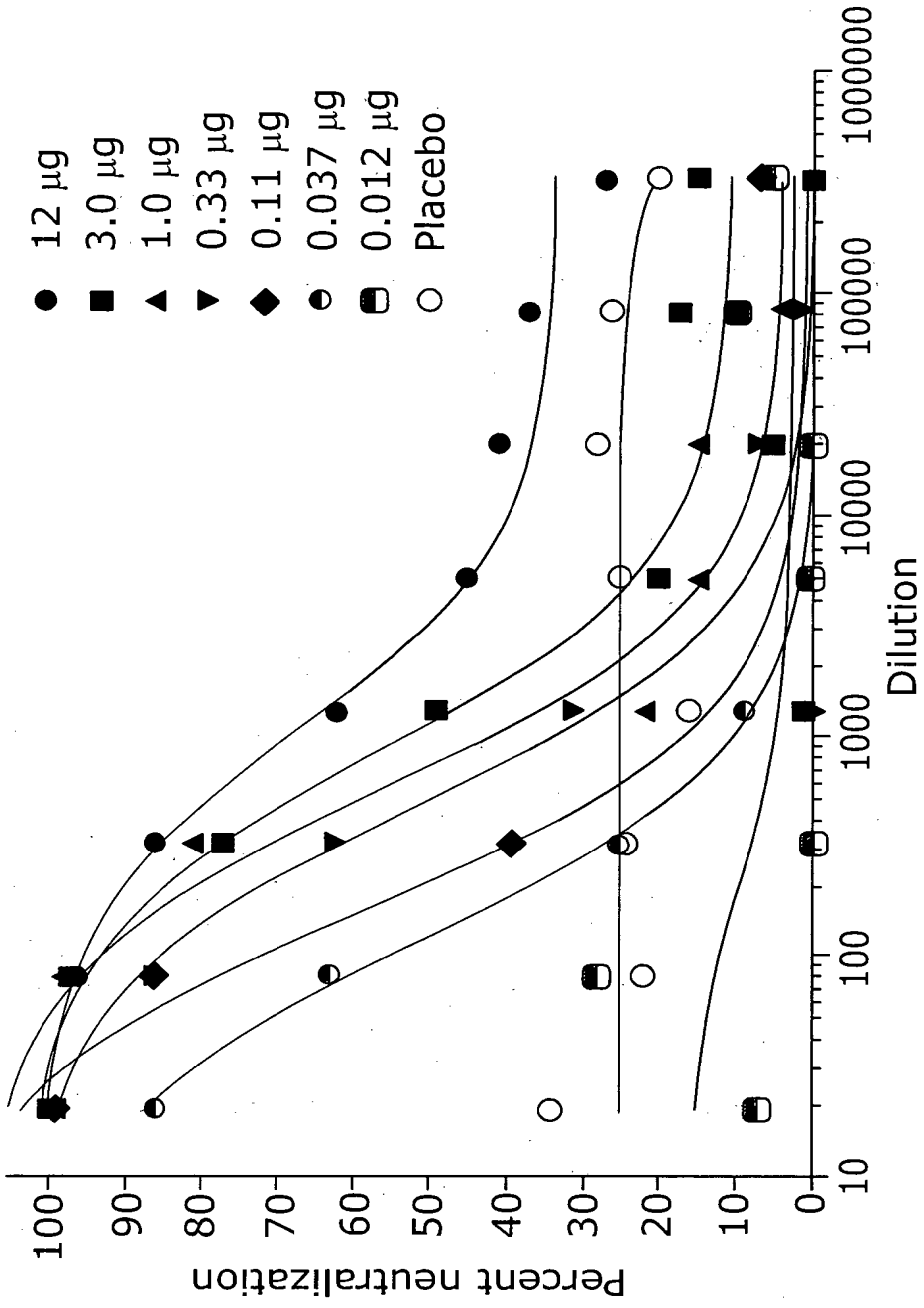

differential weight fraction (1/nm)
0.6
0.4
0.2
Distribution Analysis
Cumulative weight fraction
Differential rms radius
Cumulative weight fraction
0.8
0.6
0.4
0.2
20.0
30.0
40.0
50.0
rms radius (nm)

Figure 18



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1371	807.6	380.7	456.1	179.9	142.0

Figure 19



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1029	819.1	556.9	477.3	158.1	118.0

Figure 20

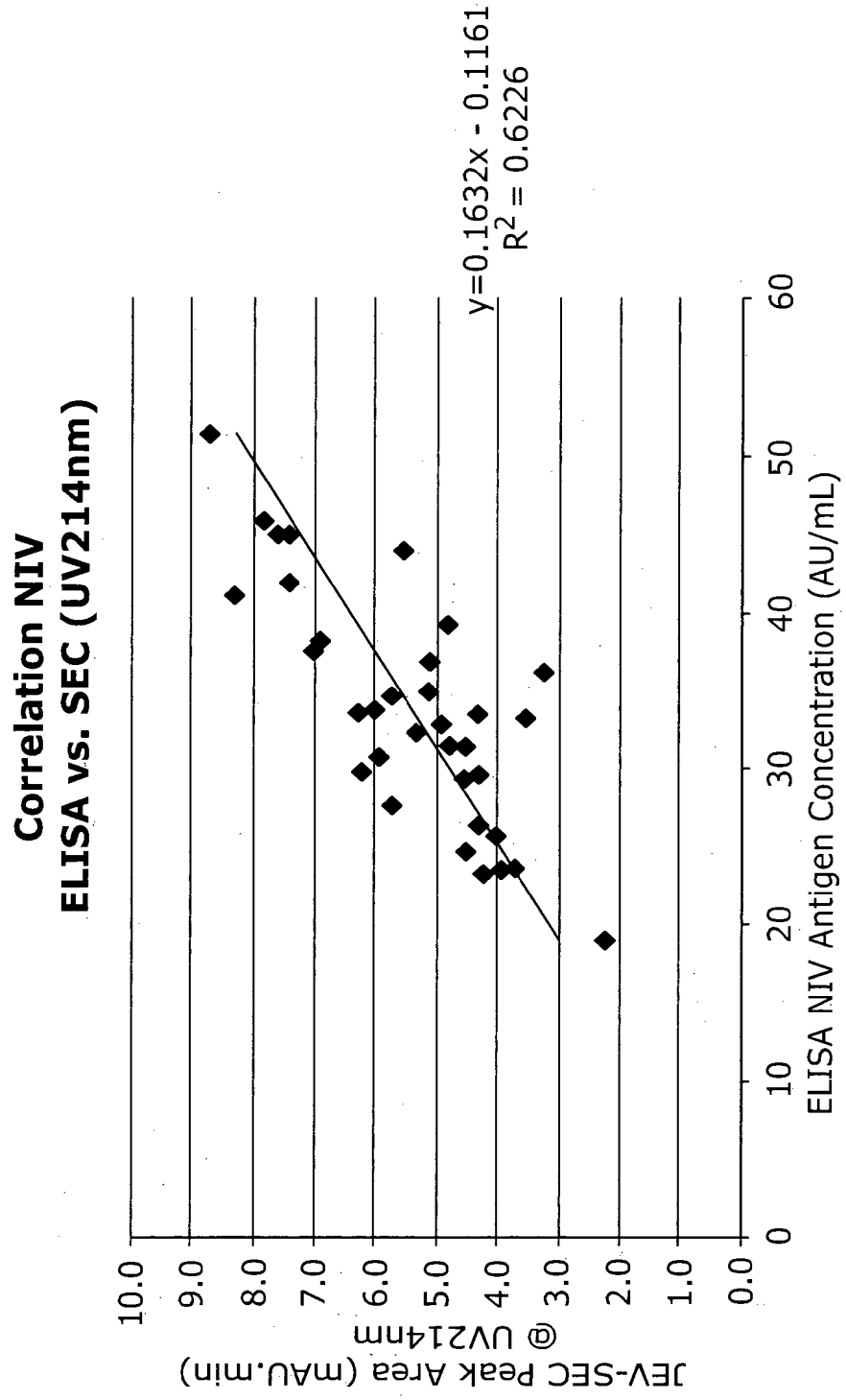

Correlation NIV
ELISA vs. SEC (UV214nm)
y=0.1632x - 0.1161
R2 = 0.6226
JEV-SEC Peak Area (mAU.min) @ UV214nm
ELISA NIV Antigen Concentration (AU/mL)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
10
20
30
40
50
60

Figure 21

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/082664

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 C07K14/005
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	B. D. COX ET AL: "Predicting Zika virus structural biology: Challenges and opportunities for intervention", ANTIVIRAL CHEMISTRY & CHEMOTHERAPY., vol. 24, no. 3-4, 1 August 2015 (2015-08-01), pages 118-126, XP055328128, GB ISSN: 0956-3202, DOI: 10.1177/2040206616653873 the whole document ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2017	10/04/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lewis, Birgit

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987, ISSN: 0264-410X [retrieved on 2001-08-14] cited in the application the whole document	1-17
Y	US 8 765 148 B2 (WIZEL BENJAMIN [AT] ET AL) 1 July 2014 (2014-07-01) cited in the application column 1	13,14
X,P	WO 2016/145149 A1 (THE US SECRETARY OF THE ARMY ON BEHALF OF THE WALTER REED ARMY INST OF) 15 September 2016 (2016-09-15) page 5, lines 8-17 paragraphs [0037] - [0040] paragraph [0048] claims 1-19	1-12,15
E	WO 2017/009873 A1 (BHARAT BIOTECH INT LTD) 19 January 2017 (2017-01-19) examples 1, 2,3,5-7	1-12,15
A	US 6 309 650 B1 (KIM HYUN SU ET AL) 30 October 2001 (2001-10-30) cited in the application examples 3-5; tables 5,6	1-17
X,P	CN 105 749 268 A (SINOVAC RES & DEV CO LTD) 13 July 2016 (2016-07-13) the whole document	1-8, 10-12,15
X,P	RAFAEL A. LAROCCA ET AL: "Vaccine protection against Zika virus from Brazil", NATURE, vol. 536, no. 7617, 28 June 2016 (2016-06-28), pages 474-478, XP055314483, United Kingdom ISSN: 0028-0836, DOI: 10.1038/nature18952 cited in the application abstract page 475, right-hand column, last paragraph - page 476, left-hand column, paragraph 1	1-12, 15-17

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/082664

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 8765148 B2	01-07-2014	EP 2536427 A1	26-12-2012
		US 2012308618 A1	06-12-2012
		US 2015216967 A1	06-08-2015
		WO 2011101465 A1	25-08-2011
WO 2016145149 A1	15-09-2016	NONE	
WO 2017009873 A1	19-01-2017	US 2017014502 A1	19-01-2017
		WO 2017009873 A1	19-01-2017
US 6309650 B1	30-10-2001	AU 748730 B2	13-06-2002
		CA 2301000 A1	11-03-1999
		CN 1272879 A	08-11-2000
		DE 69837211 T2	06-12-2007
		DE 122009000037 I1	05-11-2009
		DK 1025209 T3	25-06-2007
		DK 1604685 T3	18-06-2012
		EP 1025209 A1	09-08-2000
		EP 1604685 A2	14-12-2005
		ES 2281929 T3	01-10-2007
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		JP 4161017 B2	08-10-2008
		JP 2001514844 A	18-09-2001
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		NL 300391 I1	03-08-2009
		NZ 503522 A	29-06-2001
		US 6309650 B1	30-10-2001
		WO 9911762 A1	11-03-1999
CN 105749268 A	13-07-2016	NONE	

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	National Stage of PCT/EP2016/082664
Confirmation No.:	Not Yet Assigned
Filed:	Concurrently Herewith
For:	ZIKA VIRUS VACCINE
Examiner:	Not Yet Assigned
Art Unit:	Not Yet Assigned

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please amend the Specification as follows:

Please add the following new section following the title on page 1:

RELATED APPLICATION

This Application is a national stage filing under 35 U.S.C. § 371 of International Patent Application Serial No. PCT/EP2016/082664, filed December 23, 2016, the contents of which is incorporated herein by reference in its entirety.

REMARKS

Applicant has amended the specification to add a section containing Related Application information. No new matter has been added.

The pending application is believed to be in condition for substantive examination.

Dated: June 14, 2018

Respectfully submitted,

By: /John R. Van Amsterdam/
John R. Van Amsterdam
Registration No.: 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Filer:	John R. Van Amsterdam/Trish McDonald
Attorney Docket Number:	I0422.70125US00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
NATIONAL STAGE FEE	1631	1	300	300
NATL STAGE SEARCH FEE - REPORT PROVIDED	1642	1	520	520
NATIONAL STAGE EXAM - ALL OTHER CASES	1633	1	760	760
Pages:				
NATL STAGE APPL SZ FEE PER 50 PGS >100	1681	1	400	400
Claims:				
Miscellaneous-Filing:				
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1980**

Electronic Acknowledgement Receipt

EFS ID:	32913495
Application Number:	16063007
International Application Number:	PCT/EP2016/082664
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Customer Number:	23628
Filer:	John R. Van Amsterdam/Trish McDonald
Filer Authorized By:	John R. Van Amsterdam
Attorney Docket Number:	I0422.70125US00
Receipt Date:	15-JUN-2018
Filing Date:	
Time Stamp:	15:43:41
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1980
RAM confirmation Number	061818INTEFSW15463900
Deposit Account	232825
Authorized User	Wolf Greenfield

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.492 (National application filing, search, and examination fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal of New Application	I042270125US00-TRN-JRV.pdf	137923 ab4df36ceb6f1ed27d515630baf24dc0bf05f257	no	3
Warnings:					
Information:					
2	Application Data Sheet	I042270125US00-ADS-JRV.pdf	174249 77077e6cd37698506b379f9ba913699ffc191d71	no	7
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
3	Documents submitted with 371 Applications	I042270125US00-301-JRV.pdf	59977 48254ee3ac63a6f67e24ca807a14cf1bb3458b0d	no	2
Warnings:					
Information:					
4	Documents submitted with 371 Applications	I042270125US00-304-JRV.pdf	63982 d55e37c54aaf6ffbb2d26c1827ddc5b94e6c361b	no	1
Warnings:					
Information:					
5	Drawings-only black and white line drawings	I042270125US00-ISR-JRV.pdf	162837 47a917d30ae8482ec72ade7391ac82a633ff339d	no	5
Warnings:					
Information:					
6	Documents submitted with 371 Applications	I042270125US00-OPIN-JRV.pdf	318587 cfdf8bc63af6060ce7579a08512a2ca6e77b5494	no	7
Warnings:					

Information:					
7		I042270125US00-371APL-JRV.pdf	8552396 59dc6e933bedc0884ab03d0723ee79ea860cad04	yes	130

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Abstract	1	1
	Specification	2	102
	Claims	103	104
	Drawings-other than black and white line drawings	105	127
	Documents submitted with 371 Applications	128	130

Warnings:					
Information:					
8		I042270125US00-PAMN-JRV.pdf	103592 0cdf28c5fe8019125a8ae9678f5ada73e61fdc70	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:					
Information:					
9	Sequence Listing (Text File)	I042270125US00-SEQ-JRV.txt	466172	no	-
Warnings:					
Information:					
10	Fee Worksheet (SB06)	fee-info.pdf	37199 e4643b556b36fd6b73145bb98eb994363cc4ecb8	no	2

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

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PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

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1. The following indications appeared on record concerning:

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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

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Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
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TITLE: ZIKA VIRUS VACCINE

APPLICANT: VALNEVA AUSTRIA GMBH

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(54) **Title**: ZIKA VIRUS VACCINE

(57) **Abstract**: Described herein are Zika virus vaccines and compositions and methods of producing and administering said vaccines to subjects in need thereof.

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Bescheinigung	Certificate	Attestation
Die angehefteten Unterlagen stimmen mit der als ursprünglich eingereicht geltenden Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.	The attached documents are exact copies of the text in which the European patent application described on the following page is deemed to have been filed.	Les documents joints à la présente attestation sont conformes au texte, considéré comme initialement déposé, de la demande de brevet européen qui est spécifiée à la page suivante.

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
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Bezeichnung der Erfindung / Title of the invention / Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, oder falls die Anmeldung in einer Nicht-Amtssprache des EPA eingereicht wurde, siehe Beschreibung bezüglich ursprünglicher Bezeichnung.
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VIRUS PURIFICATION

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VIRUS PURIFICATION

FIELD OF INVENTION

The disclosure relates to methods for the purification of viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate.

The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site http://www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species

mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measurements involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can very efficiently be separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely void of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus and Chikungunya virus.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figure 7: Alignment (blue shading: % identity, aa), E-protein.

Figure 8a: Overview of an efficient Zika virus purification process.

Figure 8b: Overview of an efficient inactivation step.

Figure 9: SEC-HPLC of individual 30x concentrated Zika harvest prior PS treatment

Figure 10: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment

Figure 11: Preparation of the sucrose gradient.

Figure 12: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 13: Schematic separation of Virus (ChikV as an example) and PS/HPC using the optimized 3-layer sucrose gradient centrifugation process.

Figure 14: Correlation between JEV Antigen content in NIV analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

Figure 15: A silver-stained SDS-PAGE gel showing the protein content of the virus preparation following different steps of the process of the invention (defined in the table next to the figure). The SGC purified pool consisting of SGC fractions F7-F11 is shown in lane 12.

Figure 16: The SEC area (mAU*min; right axis) and TCID50 results (log TCID50/mL; left axis) of ChikV production harvests before and after PS treatment is shown. The grey portions of the bars indicate large losses in SEC area following PS treatment, but no corresponding change in the total number of infectious particles (indicated by black portions of the bars) (A); SEC profile of virus preparation before and after PS addition, showing a complete removal of large size virus aggregates by PS treatment as well as a reduction in host cell proteins (HCP) and LMW impurities (B).

Figure 17: Relative amounts of ChikV particles and other components by SEC-HPLC analysis at the different steps of the process of the invention including, from top to bottom: crude harvest (a) are shown; 10X concentrated harvest; diafiltrated harvest; PS treated material; CC700-treated material and SGC purified pool.

Figure 18: Comparison of different sucrose gradients (a: gradient 1: 50%, 10% sucrose, b gradient 2: 50%, 35%, 10% sucrose, c: gradient 3: 50%, 25%, 10% sucrose, d: gradient 4: 50%, 35%, 15%, 10% sucrose) with ChikV particles.

Figure 19: Comparison of two different ZikaV harvest schedules.

Figure 20: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column.

Figure 21: SEC-MALLS analysis of inactivated ZikaV.

Figure 22: Cumulative particle size distribution of Zika NIV.

Figure 23: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse serum. The number of plaques without serum is set to 100%. The EC50 is calculated with the 3-parameter method.

Figure 24: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse serum. The number of plaques without serum is set to 100%. The EC50 is calculated with the 3-parameter method.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are virus vaccines and compositions comprising an inactivated virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely void of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus and Chikungunya virus.

Disclosed herein are downstream processes for purifying virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for propagation on a particular cell substrate or for a partial / fully cell substrate adapted virus particle.

Aspects of the invention provide processes for the purification of infectious virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 1.6 mg/ml (for e.g. Chikungunya) or about 2 mg/ml (for e.g. Zika).

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In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the detection limit in particular below the detection limit in the final drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of 100 kDa.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may by optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have

reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In some embodiments, the virus belongs to a virus family selected from the group consisting of Paramyxoviridae, Orthomyxoviridae, Flaviviridae, Filoviridae, Arenaviridae, Rhabdoviridae, and Coronaviridae. In some embodiments, the virus belongs to a virus family selected from the group consisting of Togaviridae (being live or inactivated), such as alphaviruses, or Flaviviridae (being live or inactivated). In some embodiments, the virus is a virus of the family Flaviviridae, i.e. a flavivirus. In other embodiments, the virus is a Zika virus or Yellow Fever virus. In preferred embodiments, the virus is a Zika virus. In a most preferred embodiment, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the filtration of step in (b)(ii) of claim 1 is performed using a filter having a pore size equal to or greater than 1 μm. In some embodiments, the filter has a pore size equal to or greater than 0.2 μm. In a preferred embodiment, the filter has a pore size of 0.2 μm.

In some embodiments, the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian cell line. In some embodiments, said cell line is EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred

embodiment, the composition is a vaccine. In one embodiment, the composition or vaccine is directed against Chikungunya virus. In one embodiment, the composition or vaccine is directed against a flavivirus. In one embodiment, the composition or vaccine is directed against Yellow Fever virus. In one embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the virus particles obtainable by any of the processes described herein for treating and/or preventing a viral infection. In one embodiment, the viral infection is caused by Chikungunya virus. In one embodiment, the viral infection is caused by a flavivirus. In one embodiment, the viral infection is caused by Yellow Fever virus. In one embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on the average for their age, sex, and ethnicity. This is

a result of the brain failing to undergo proper embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has a RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the attenuated form of ChikV is derived from the LR2006-OPY1 ChikV infectious clone (La Reunion isolate). In some embodiments, the attenuated form of ChikV is the Δ5nsP3 mutant as described by Hallengärd et al. (Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice (2014) Journal of Virology 88(5):2858–2866) or an immunogenic variant thereof. The immunogenic variant of the Δ5nsP3 ChikV mutant is herein defined as having at least 80% sequence identity to the nucleotide sequence of the Δ5nsP3 mutant sequence, especially at least 81%,

82%, 83%, 84%, 85%, 86%, 87%, 88% 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least 99% sequence identity.

In some embodiments, the process of the invention results in an enrichment of infectious virus particles from the crude harvest comprising infectious virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

Another aspect of this invention is the realization that by using an approach of a type of vaccine (i.e. the Japanese Encephalitis Vaccine (JEV) with tradename IXIARO®, see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) which the regulatory agencies have seen and licensed before, it may be possible to very quickly develop a Zika or Chikungunya vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, had to be complemented by further inventions disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines are their avoidance of any protamine salt (SEQ ID NO: 1) in the final drug substance by introducing an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the VERO (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation need to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0,01 MOI (multiplicity of infection), to allow for viral production, prior to passaging. In some embodiments, the Vero cells are incubated for

at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. In order to minimize the adoption of Zika virus strain to Vero cells, it was found that Vero cell could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days 2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 μm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant," is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA's. The fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in experimental part. It was surprisingly found that addition of a 10% sucrose virus containing fraction to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) was able to efficiently separate protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, e.g. a Zika virus, Yellow Fever virus or Chikungunya virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the

terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agent for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38

days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphohetain. A preferred inactivation is the inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments,

the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time.

It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

It is believed that the herein described improved process (comprising the PS treatment in combination with the optimized sucrose gradient) is applicable and efficient to any virus purification and in particular efficient for any RNA type virus (such as the herein described Zika and Chikungunya and yellow fever viruses) of similar size (i.e. about 50 to 100 nm). Furthermore, it is believed that the combination of the PS treatment with the optimized sucrose gradient (allowing for a complete (or almost complete) separation of PS provides a very efficient virus purification in the very high range, e.g. above 70%, more preferably 75%, 80% or 90%, even more preferably 95%. It is believed that the complete reduction of PS in the virus fraction through the process of the invention allows a very efficient inactivation with almost no or very low viral loss e.g. below 30%, more preferably less than 25%, 20% or 10% loss, even more preferably less than 15% loss.

Any of the methods or uses described herein may be for the prevention of a virus infection in a subject. As used herein, the terms "prevent," and "preventing," include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the virus. As used herein, antigen(s), such as an inactivated virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck trunk, upper arms; headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is likely associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus antibodies). In some embodiments, the therapeutically effective amount

is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) present in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)).

Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 70). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 69). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its

entirety). In some embodiments, the adjuvant is IC31®, i.e. $KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 72), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccine to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the

invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms hall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by

reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

SEQUENCES

The amino acid sequence of a typical form of protamine is provided by SEQ ID NO: 1: PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR.

Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome (SEQ ID NO: 2)

GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCA
ACAGGTTTTATTTGGATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGG
AGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAA
GAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCAATTCTAGCCTT
TTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAA
AAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAAT
GCTAGGAAGGAGAAGAAGAGACGGGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGAC
CACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACG
ATGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTTATATACAGATCATGG
ATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAA
CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCAC
AAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCT
GCAAACGCGGTCGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAA
ATTGGATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCT
CAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGCATCAGGT
GCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGTTGATATTGTCT
TGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTT
ACAACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCAGACAT
GGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAAT
ATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGG
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KU509998.1 Zika virus strain Haiti/1225/2014, Haiti, complete genome (SEQ ID NO: 5)

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KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome (SEQ ID NO: 7)

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KU681082.3 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, complete genome (SEQ ID NO: 8)

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KU744693.1 Zika virus isolate VE_Ganxian, China, complete genome (SEQ ID NO: 10)

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LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome

(SEQ ID NO: 11)

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>Chimeric Asian ZIKV Sequence H/PF/2013 polyprotein flanked with consensus sequence from other strains (SEQ ID NO: 13)

GTTGTTGATCTGTGTGAATCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCA
ACAGGTTTTATTTTGGATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCG
GAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGA
AGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCTAGCCT
TTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAA
AAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAAT
GCTAGGAAGGAGAAGAAGAGACGAGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGAC
CACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACG
ACGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTTATATACAGATCATGG
ATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAA
CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCAC
AAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCT
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AGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGA
GAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGA
CACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCC
GAAGCCACCCTGGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTT
TCAGATTTGTATTACTTGACTATGAATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGAC
ATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAACTCCACACTGGAACAACAAAGAAGCACT
GGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAG
CAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCC
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TGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGA
GGTACAGTACGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAA
CTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTA
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GCAGCAGTCAAGCAAGCCTGGGAAGATGGTATCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAA
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CGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGCCTGTG
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TAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACA
GCTTTCTTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAG
ATTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACA
GTGATCTAGGCTACTGGATTGAGAGTGAGAAGAATGACACATGGAGGCTGAAGAGGGCCCATCTG
ATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGACAGATGGAATAGAAGAGA
GTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCTACA
GGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGC
ACTAAGGTCCACGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAG

CGGAAGGGTGATCGAGGAATGGTGCTGCAGGGAGTGCACAATGCCCCCACTGTCGTTCCGGGCTA
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GTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGTGCTTGTGATTCT
GCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCGATG
GCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTTG
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CATGCTGCTGGCCTTGGCCTCGTGTCTTTTGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGAT
GGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCACTGAT
AACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGG
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GGTGGGACTGCTGTTGCTCACAAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAG
CTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTCGCCAAGGCAGATATAGAGATGGCTGGGCCC
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ATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAG
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CAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAGACTGCTA
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AAAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAG
GATCTGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCA
GCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGA
CAAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAACTTCAGGATCTCCAATC
CTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGTTA
TGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGCCTTCGA
TGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTT
CTTCCTGAAATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGG
GTTGTCGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCAGTC
AATGTCACCCACTCTGGAACAGAAATCGTCGACTTAATGTGCCATGCCACCTTCACTTCACGTCTA
CTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCACTTCACAGATCCC
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CATGACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACAC
CGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTCTGGAA
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CCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGACTGACGAAGACCATGCACACTGGCT
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TGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACC
TTTGTGGAACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGA
ATAACCTACACAGATAGAAGATGGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAG
TGTGCCGGCAGAGGTGTGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGAC
GCCAGAGTTTGTTCAGATCATGCGGCCCTGAAGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGG
AGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACACATGACAGAGAGATTCCAGG
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GTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAGCCAGAAAAGCAAAGATCTCCCCAGGACAAC
CAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTACCGCCAATGAACTCGGA
TGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAACCA
TAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAA
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TGGCCACGCAAGCTGGAGTGTTGTTTGGTATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTG
GAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACCCCTGACCCTAATAGTGGCCATCA
TTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGCGTGCTGCCC
AGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATT
GACACAATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGC
CGTCTCCAGCGCCATACTGTCGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAG
CGGCAACTTCCACTTTGTGGGAAGGCTCTCCGAACAAGTACTGGAACTCCTCTACAGCCACTTCAC
TGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTACACAGTAACAAGAAACG
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CTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAG
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CACTATGATGGAAACCCTGGAGCGACTGCAGCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCAC
TCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCGAAAAGCAACACCATAAAAAGT
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AGATCATTGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAG
AACCACCCATATAGGACATGGGCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTC
CTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAAACCCTGGGATGTGGTGACTGGAGTCACAGG
AATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAGGAAAAAGTGGACACTA
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AGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGC
AATGCAGCATTAGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGA
ACGATCCAAGGTTCTGGGCTCTAGTGGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCA
GAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGAAACAAGGGGAATTTGGAAAGGCCAAG
GGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGAAGCCCTTGGATTC
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AAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGACATTATTTCGAGACAAGACCAAA
GGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGCAACTCATTC
GGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAA
GTGACCAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGA
TTGCGTTGTGAAGCCAATTGATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAA
AGTTAGGAAGGACACACAAGAGTGGAAACCCTCAACTGGATGGGACAACTGGGAAGAAGTTCCG
TTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCCATTGTGGTTCCCTGCCGC
CACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGGAGAC
TGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCG
ACTGATGGCCAATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTG
GTCAATCCATGGAAAGGGAGAATGGATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGT
GGATTGAGGAGAACGACCACATGGAAGACAAGACCCCAGTTACGAAATGGACAGACATTCCCTAT
TTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGCGCACCACCTGGGC
TGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGG
ACTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCAC
CAATCTTAGTGTTGTCAGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCC
CCCCAGGAGAAGCTGGGAAACCAAGCCTATAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGC
CATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACCCCACGCGCTTGGAGGCGCAGG
ATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGATCAGCTGT
GGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAGACCCCCCGGAAAACGCAAAACAGCATATTG
ACGCTGGGAAAGACCAGAGACTCCATGAGTTTCCACCACGCTGGCCGCCAGGCACAGATCGCCGA
ATAGCGGCGGCCGGTGTGGGGAAATCC

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E. (SEQ ID NO: 14)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNN
KHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGAL
EAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCK
VPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGK
AFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGL
NTKNGSISLTCLALGGVMIFLSTAVSA
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E. (SEQ ID NO: 15)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNN
KHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGAL
EAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGRDGPCK
VPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGK
AFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGL
NTKNGSISLTCLALGGVMIFLSTAVSA
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 16)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVIIGSQIISGMIVNDXXXXXXXXNRAEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYA
GTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWH
RSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIIIQIFGAAFKSLFGGMSWFSQILIG
TLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA
ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 17)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMXXXXXGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYA
GTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWL

KKGSSIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIG
TLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 18)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYY
LTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTA
LAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGT
DGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 19)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYY
LTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTA
LAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGT
DGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 20)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYY
LTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTA
LAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGT
DGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 21)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYY
LTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTA
LAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGT
DGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 22)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYA
GTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWH
RSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIG
TLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 23)

60

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMTVNDIGYETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYA
GTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E. (SEQ ID NO: 24)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMTVNDIGYETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYA
GTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 25)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDTGYETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYA
GTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 26)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYY
LTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTA
LAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E. (SEQ ID NO: 27)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIGYETDEDRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTM
NNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAG
ALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGP
CKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTI
GKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVW
LGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL (SEQ ID NO: 28)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIGYETDEDRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTM
NNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAG
ALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGP
CKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTI

GKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVW
LGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus] (SEQ ID NO: 29)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIGYETDEDRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTM
NNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAG
ALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGP
CKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTI
GKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVW
LGLNTKNGSISLTCLALGGVMIFLSTAVSA

KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E. (SEQ ID NO: 30)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGT
LLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 31)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGT
LLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1 (SEQ ID NO: 32)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.] (SEQ ID NO: 33)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E. (SEQ ID NO: 34)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY

YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E. (SEQ ID NO: 35)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E. (SEQ ID NO: 36)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 37)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.] (SEQ ID NO: 38)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 39)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E. (SEQ ID NO: 40)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 41)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E. (SEQ ID NO: 42)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E. (SEQ ID NO: 43)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 44)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E. (SEQ ID NO: 45)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT

ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 47)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 48)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E. (SEQ ID NO: 49)
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDM
ASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENL
EYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYL
TMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTAL
AGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTD
GPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGS
TIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLM
WLGLNTKNGSISLMCLALGGVLIFLSTAVSA

ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 50)
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDM
ASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENL
EYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYL
TMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTAL
AGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTD
GPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGS
TIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLM
WLGLNTKNGSISLMCLALGGVLIFLSTAVSA

103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus].

103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E. (SEQ ID NO: 51)

65

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDM
ASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENL
EYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYL
TMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTAL
AGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTD
GPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGS
TIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLM
WLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 52)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGTQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E. (SEQ ID NO: 53)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGTQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 54)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 55)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus] (SEQ ID NO: 56)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS

GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNAKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus] (SEQ ID NO: 57)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNAKNGSISLMCLALGGVLIFLSTAVSA

isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 58)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 59)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNGTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 60)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGT
LLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 61)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E. (SEQ ID NO: 62)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY

YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 63)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRS
GSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTL
LVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 64)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDI
ASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENL
EYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYL
TMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTAVVLGSQEGAVHTAL
AGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTD
GPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGS
TIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLM
WLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 65)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMIVNDXGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLY
YLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHT
ALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXR
SGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGT
LLVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E. (SEQ ID NO: 66)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMLVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDL
YYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVH
TALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTVEGQYG
GTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWH
RSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGT
LLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSG

isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 67)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPE
NLEYRIMLSVHGSQHSGMLVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDL
YYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVH
TALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTVEGQYG
GTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWH
RSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGT
LLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSG

ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E. (SEQ ID NO: 68)
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSD
MASASRCPTQGEPSLDKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENL
EYRIMLPVHGSQHSGMIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYL
TMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTAL
AGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQSAGTD
GPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSG
STIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLL
VWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E. (SEQ ID NO: 69)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISD
MASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPEN
LEYRIMLSVHGSQHSGMIVNDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNN
KHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGAL
EAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCK
VPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGK
AFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGL
NTKNGSISLTCLALGGVMIFLSTAVSA

In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NO: 14-69. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Or., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977; and Altschul et al., J. Mol. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were applied to adapt on ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 8a+b).

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):
- 5% CO2, 35°C, MEM+2mM L-Glutamine+10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01
- Virus Production without serum

- 5% CO2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Definitions & Abbreviations

BSA	Bovine serum albumin
HPLC	High performance liquid chromatography
JEV	Japanese Encephalitis Virus
MALS	Multi angle light scattering
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
rms radius	Root mean square radius
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGP	Sucrose gradient purified
ZikaV	Zika Virus
rMSB	research master seed bank
p.i.	Post infection
SGC	Sucrose gradient chromatography
PS	Protamine sulfate

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (SEQ ID NO: 13). A sample was obtained from European Virus Archive EVAg. Based on this material, a research master seed bank (rMSB) was prepared on vero cells as the cell substrate.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significant higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with day 3 harvest the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest. The calculated titers per plaque assay are summarized in the list below:

Log 10 PFU/mL

sample day2 7.02

harvest day 3 6.66

harvest day 5 6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 19. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22 °C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 9 for SEC_HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 10 for SEC_HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 12). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

PAT018/EP/PR3

Samples of individual 30x concentrated harvests day 2, 3, 5 and 7 were analysed before and after PS treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 1. The virus peak recovery by SEC. HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 1: ZikaV Recovery by SEC-HPLC and Plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x +PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x +PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles.

Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different density. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus particles from residual contaminants as demonstrated for ChikV, see Figure 13. The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 2.

Table 2: Individual layers/volumes for a centrifugation in bottle

~40 mL PS treated harvest load Load
15 mL 15 % Sucrose solution J
15 mL 35 % Sucrose solution I
20 mL 50 % Sucrose solution H
90 mL total volume

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle (see Figure 11). Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 11. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4 °C. The prepared SG bottles were carefully transferred into the pre-cooled rotor (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers). The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no break/deceleration activated. In case a different centrifuge system with a different rotor is

used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle.

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL) and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 12. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51mL in a PETG container. A volume of 1% formaldehyde (10.000ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25°C) for a minimum

of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criteria for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution is monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. In short,

a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25 °C, is used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 14.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gentle mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 3).

Table 3: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	**JEV**	**Zika**
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 20. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 21) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6nm and ~49nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 22).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Active ZikaV was analyzed by plaque assay throughout the process.

Sample	Pfu/mL
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$
Harvest day 5 (filtered)	$1.5x10^8$
Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD
Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly,

the Benzonase treated samples did not sure any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell proteins was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well were seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5%CO2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicates. The target virus (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were dilute to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were incubated at 35°C with 5%CO2 for 1 hour. The cell culture medium were aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plated were rocked back and forth 5

times before incubated for 2 hours at 35°C with 5%CO2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5%CO2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 23 and 24). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, http://dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are

optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) with anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV Vaccine. The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Example 2: Development of a purification process for live attenuated Chikungunya virus vaccine produced in Vero cells

A downstream process was developed for the purification of infectious Chikungunya virus particles whereby non-infectious virus particles and aggregates are removed by the addition of protamine sulphate. The unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Chikungunya Virus (ChikV) as follows:

A downstream purification process for the attenuated Chikungunya virus mutant "Δ5nsP3" (as described by Hallengärd et al., 2014, supra) produced under standard cell culture conditions in Vero cells was developed. Briefly, the downstream process consists of crude harvest filtration followed by concentration and diafiltration on a tangential flow filtration (TFF) system. DNA and host cell proteins were reduced by precipitation with protamine sulphate and by batch adsorption, respectively. Sucrose density gradient centrifugation was done as a final polishing step. Out of 16 roller bottles 1 x 1012 total PFU were purified with an overall DSP process yield of 10 - 15 % (~1 log10 TCID50 loss). Sucrose gradient pool samples were characterized with regard to product-related impurities, such as hcDNA, HCP and endotoxins and met safety criteria.

Harvest of Vero cell culture medium containing ChikV Δ5nsP3

ChikV Δ5nsP3 was grown on Vero cells in roller bottles. A first harvest was performed after 24 hours post infection (hpi; day 1 harvest) and stored at 2-8°C until further processing. After the first harvest, fresh medium was added and the roller bottles were returned to the incubator. A second harvest was done after 48 hours post infection (day 2 harvest) and stored at 2-8 °C.

Filtration of crude cell culture harvest

At both harvest timepoints the crude harvest was immediately filtered using a 0.2 µm filter capsule (GE ULTA™ CG, 2 inch). The filtered harvest after 48 hpi was pooled together with the 24 hpi harvest and the pooled filtered harvest material was immediately further processed by ultrafiltration.

Purification of ChikV Δ5nsP3 by tangential flow filtration (TFF)

The pooled filtered harvest material was further processed by tangential flow filtration (TFF) in order to concentrate the harvest, reduce host cell proteins and replace the depleted cell

culture medium with a defined buffer system (buffer exchange). A Millipore TFF system (Millipore Pellicon II mini membrane holder) equipped with a 100 kDa cutoff PES membrane module (Pellicon2 Biomax, 1000 cm^2) was used for concentration and buffer exchange. A Pellicon2 Biomax membrane module was mounted on the Pellicon II mini filter holder and the device was connected to a peristaltic pump. The system was first rinsed with ultra-pure water and then sanitized by recirculation of 0.1 M NaOH for 60 min. In case the system was not used immediately, it was stored in 0.1 M NaOH until use. Prior to use the system was rinsed with 1 L of RO-water followed by buffer A until the permeate pH value was constant at pH 7.4 ± 0.2.

Adjustment of the ChikV Δ5nsP3 harvest (pH, salt)

The pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris and 150 mM NaCl using stock solutions of both components (see Table 1). This adjustment was done to increase buffering capacity and to reduce unspecific adsorption to the membrane. The necessary volumes of stock solutions D (1 M Tris, pH 7.4) and E (4.5 M NaCl) were calculated as follows:

Volume of stock solution D (1 M Tris, pH 7.4) added to pooled harvest = Volume of pooled filtered harvest / 40

Volume of stock solution E (4.5 M NaCl) added to pooled harvest = Volume of pooled filtered harvest / 30

4 L harvest obtained from 20 RB (850cm^2) would require addition of 100 mL stock solution D (1 M Tris, pH 7.4) and 133 mL stock solution E (4.5 M NaCl).

The calculated volumes of stock solution D and Buffer E were added to the pooled filtered harvest under gentle stirring. The adjusted harvest was then stirred using a magnetic stirrer for 5 minutes at room temperature.

Concentration and diafiltration of the ChikV Δ5nsP3 harvest by TFF

In a first step the adjusted harvest material was concentrated approximately 10 fold. The feed flowrate was approximately 220 mL/min. The transmembrane flux at a transmembrane pressure of approximately 0.6 bar was in the range of 90 ± 5 mL/min per 1000 cm^2 membrane. After concentration, the cell culture medium was exchanged against 25

mM Tris, 150 mM NaCl, pH 7.5 by continuous diafiltration with 6 volume exchanges. The diafiltration buffer was supplied to the feed vessel from a measuring cylinder by a second peristaltic pump set to a flowrate of approximately 90 mL/min. Minor flowrate adjustments of the second peristaltic pump in the range of ± 10 mL/min were done manually to ensure a constant volume of harvest in the feed vessel. After 6 volume exchanges, diafiltration was stopped. The liquid remaining in the membrane module was recovered by pumping the module empty with air.

Sucrose addition to diafiltrated ChikV Δ5nsP3 material

After diafiltration, sucrose stock solution H (50% (w/w) sucrose solution) was added to the diafiltrated material to achieve a final sucrose concentration of 10% (w/w). The volume of buffer H was calculated as follows:

Volume of stock solution H added (mL) = Volume (mL) of diafiltrated ChikV material x 0.25 (dilution factor = 1:4) (i.e., final sucrose concentration is 10%)

Example: 400mL diafiltrated ChikV solution would require addition of 100mL stock solution H (50% sucrose).

The calculated volume of solution H was added to the diafiltrated ChikV Δ5nsP3 material under gentle stirring and the solution was then stirred using a magnetic stirrer for a further 5 minutes at room temperature. (At this stage of the process the material can be either immediately further processed or stored frozen (< -65°C, hold step).)

DNA reduction by protamine sulphate precipitation

A DNA precipitation step using protamine sulphate (PS) was performed to reduce hcDNA. Protamine sulphate stock solution L (50 mg/mL PS in PBS) was added to the diafiltrated ChikV Δ5nsP3 material to a final nominal concentration of ~1.6 mg/mL. The necessary volume of stock solution L was calculated as follows:

Volume of stock solution L (50 mg/mL PS) added = Volume of diafiltrated ChikV Δ5nsP3 material in 10% sucrose / 31

Example: 500 mL diafiltrated ChikV Δ5nsP3 solution in 10% sucrose would require addition of 16 mL stock solution L (50 mg/mL PS in PBS).

The protamine sulphate stock solution was added while stirring the ChikV Δ5nsP3 material using a magnetic stirrer followed by incubation at 2-8°C for 30 minutes. After incubation, the DNA precipitate was not removed. The material was immediately further processed by batch adsorption with Capto™ Core 700 chromatography media.

Batch adsorption with Capto™ Core 700

To reduce HCPs, a batch adsorption step with Capto™ Core 700 (CC700) chromatography medium was performed after DNA precipitation. CC700 slurry (50% slurry in buffer A) was added directly to the protamine sulphate treated material. The required slurry volume was determined based on the volume of ChikV harvest material (d1+d2) and was calculated as follows:

Volume of CC700 slurry added to PS-treated concentrated harvest (mL) = Volume of ChikV Δ5nsP3 harvest material (mL) x 0.02 (dilution factor = 1:50) (i.e., final concentration of CC700 is 1%)

After slurry addition, the material was incubated at 4°C for 15 minutes under constant agitation using a magnetic stirrer. After incubation, the CC700 solid matter was allowed to settle by gravity for 10 minutes. The ChikV Δ5nsP3 material was then removed from the top of the solution in order to avoid blocking of the filter by the CaptoCore particles. The remaining CaptoCore particles and the DNA precipitate were then removed from the solution by filtration using a 0.2 µm Mini Kleenpak EKV filter capsule (Pall). The resulting filtrate was further processed by sucrose density gradient centrifugation.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ChikV Δ5nsP3 material. The ChikV material was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and good separation of the virus particles from residual contaminants. The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation at 500 mL scale are shown in Table 3.

Table 3. Sucrose concentrations and volumes (500 mL scale).

Solution	Volume (mL)

Harvest with 10% sucrose	360
15% sucrose	40
35% sucrose	40
50% sucrose	60
Total volume	500

Preparation of the sucrose gradient

The sucrose gradient bottles (500 mL) were prepared by underlaying the individual sucrose layers. A 3.5 mm ID plastic tube was attached to 60 cm of peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump set to a flow rate of 25 mL per minute, the ChikV Δ5nsP3 material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as a feed vessel. The first solution pumped was the ChikV Δ5nsP3 material as it had the lowest density (10% sucrose (w/w)). Following the addition of the ChikV Δ5nsP3 material, the sucrose solutions were pumped in ascending order starting with the lowest (15%), followed by the 35% sucrose solution and finishing with the highest density sucrose solution (50%). After all sucrose solutions were transferred, the plastic tubing was carefully removed in order not to disturb the layers.

Centrifugation

Prior to centrifugation a Beckman Avanti JXN-26 centrifuge equipped with rotor Beckman 10.500 was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled (4°C) rotor so as to not to disturb the sucrose layers. The bottles were centrifuged at 10,000 rpm (~18,500 rcf) at 4°C for 17-20 hours. (In case a different centrifuge system with a different rotor would be used, the necessary speed and centrifugation times would need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.)

Sucrose gradient harvest

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A 3.5 mm ID plastic tube attached to 60 cm of peristaltic pump tubing was used for harvesting the

sucrose gradient. The 500 mL bottle containing the centrifuged gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle. Using a peristaltic pump set to a flow rate of 60 mL per minute, the gradient was harvested and manually split into 5 mL fractions. A third of the bottle volume was harvested and the rest was discarded. The fractions were immediately tested by measuring UV absorbance in a plate reader as described below.

Analysis of fractions by UV absorbance and SEC-HPLC

UV absorbance measurement was used as primary method for analysis of the sucrose gradient fractions. Absorbance at 214, 280 and 260 nm was tested immediately after fractionation was completed. Briefly, a 100 µL sample of each fraction was transferred into a 96 well plate and absorbance at 214, 260 and 280 nm was measured using a plate reader. The absorbance values were plotted against the fraction number. The presence of impurities was indicated by an increase of the UV214 signal after the main peak. The fractions comprising the main peak were pooled from the peak start to the valley of the 214 nm curve. This method can be used as single method for pooling ChikV Δ5nsP3 fractions.

After identification of the virus containing fractions, the respective fractions were pooled. Pooling criteria for SGC fractions were based on UV 260 nm data, e.g. start of pooling at ~10% of peak maximum, end of pooling at ~30% of peak maximum. (Final pooling criteria at a manufacturing scale may need to be determined empirically.) The sucrose gradient pool was either stored at < -65 °C or immediately further formulated to drug substance (DS).

Size exclusion chromatography

The final pooled SGC fractions containing purified infectious ChikV Δ5nsP3 virus particles were analyzed for purity by SEC-HPLC. In brief, SEC was performed as follows: a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ChikV particles at 214 nm detection wavelength in the pooled samples. SEC-HPLC is a semi-quantitative (relative yield) and qualitative (purity) method that separates intact virus particles from virus aggregates and host

cell proteins (HCPs). The method cannot distinguish between infectious and non-infectious virus particles due to their identical retention time.

Two defined peaks were identified by SEC: the ChikV Δ5nsP3 peak and a peak corresponding to buffer components. The SGC step yield based on SEC-HPLC data for pooled fractions F6-F11 was estimated at ~70 %.The final purity of the ChikV Δ5nsP3 SGC pool, based on SEC-HPLC analysis, was estimated at >95%.

SDS-PAGE and silver stain

SDS-PAGE silver stain was performed in order to qualitatively assess sample purity throughout the purification process from the first crude harvest through SGC. Briefly, ChikV process samples analyzed by SDS-PAGE/silver stain were diluted 1:1.33 with LDS buffer and were heated to 70°C for 5 minutes. The samples were loaded onto 4-12 % Bis-Tris Gels (NuPAGE). Silver staining was done using the Silver Express staining kit (Invitrogen).

A silver-stained gel of a representative ChikV Δ5nsP3 purification is shown in Figure 15. The viral proteins E1, E2 and C are marked on the right-hand side of the gel. The final SGC pool (fraction 7-fraction 11) is shown in lane 12. Note that a defined HCP band migrating between ChikV protein E2 and C still appears after CaptoCore700 treatment that has been identified as a single band in SDS-PAGE. This impurity is removed by sucrose gradient centrifugation, but can still be seen in fractions 13 and 14 (corresponding to lanes 14 and 15 of Figure 15).

Enrichment of infectious ChikV Δ5nsP3 particles by PS treatment

Although generally used as a method of removing contaminating hcDNA, it was observed in the course of the present invention that PS treatment also removes virus aggregates and HCPs. Size exclusion chromatography (SEC-HPLC, as described above) was used to throughout the purification process to determine the purity of the ChikV virus relative to impurities which also generate UV absorption.

As can be seen in Figure 16a, treatment with PS reduces not only host cell proteins and low molecular weight contaminants of the ChikV Δ5nsP3 preparation, but also reduces the SEC area corresponding to virus products, including aggregates as indicated. A surprising finding, however, was that even a reduction of the total SEC area by 86% (in a representative experiment shown in Fig. 16B, grey portion of bars) did not result in a

concomitant reduction in infectious virus particles as measured by TCID50 (left axis). Instead, even though a large percentage of virus particles were removed by PS treatment, the infectious particles were not. This observation indicates that PS treatment selectively enriches infectious virus particles from a larger pool of total virus particles present in the crude harvest.

TCID50 was performed to quantify infectious virus particles during the course of the purification process and to assign an active virus titer to final drug substance and drug product samples. Briefly, Vero cells were seeded at 2 x 104 cells per well in 100 µL medium (EMEM with 2 mM L-Glutamine + 5 % FBS (+ 1 % antibiotic/antimycotic) in 96-well TC-treated flat-bottom plates and incubated overnight at 35°C / 5% CO2. On day two, Vero cell monolayers were infected by adding 100 µL of 1:10 serial dilutions of test samples to each of quintuplicate wells seeded with Vero cells and incubated at 35°C / 5% CO2. On day seven, plaques were counted by visualization under a microscope. The TCID50 was calculated according to the Reed & Münch endpoint calculation method (Reed, L.J.; Muench, H. (1938) A simple method of estimating fifty percent endpoints" The American Journal of Hygiene 27: 493–497).

Furthermore, electron microscopy of ChikV samples before and after PS treatment showed that not only large aggregates but also smaller non-infectious virus-like particles (basically not fully assembled particles without RNA genome) are effectively removed by PS.

Furthermore, this enrichment of infectious virus particles could also be observed when analyzing day one and day two crude harvests separately. As presented in Table 4, the SEC area (total virus particles) of the day 1 harvest remains roughly the same after PS treatment; whereas a large decrease in virus peak area is seen for the day 2 harvest. This observation was confirmed by MALLS analysis of the virus preparation, wherein it was seen that a higher percentage of virus particles were of the correct size following PS treatment. Similarly to the results shown in Fig. 16, day 1 and day 2 harvests showed no reduction in infectious particles as measured by TCID50 following PS treatment, indicating that mainly non-infectious, immature and/or aggregated virus particles are removed during the PS treatment and infectious particles are enriched in the preparation.

Table 4. Overview of the process of ChikV Δ5nsP3 purification as described in Example 1. SEC-MALLS analysis of harvests before and after PS treatment shows the removal of larger virus particles (aggregates), an effect that is particularly pronounced for day 2 harvests.

	SEC MALLS Area [mAU*min] (20-40 nm)	Infectious particles Total particles/mL TCID50 log 10	% correct size	
Harvest 1 (H1)	57	1.17E+11	49%	10.2
Harvest 1 + PS	53	1.33E+11	81%	10.0
Harvest 2 (H2)	36	4.60E+09	3%	7.9
H2 + protamine sulphate	2	8.80E+09	59%	7.9
Combined Harvests (C)	67	2.60E+10	14%	9.9
C + protamine sulphate	24	8.00E+10	72%	10.1

Finally, an overview of the relative amounts of ChikV particles and other components as measured by SEC-HPLC at various steps throughout the entire virus purification process from crude harvest (a) to the final SGC purified pool can be seen in Figure 17. In sum, not only are the vast majority of contaminants and undesired products removed by the process, ChikV particles are highly purified. As shown by the previously presented data, the final preparation is a highly enriched preparation of infectious ChikV particles.

Drug Substance (DS) Formulation

The pooled SGC fractions are diluted with DS formulation buffer M (10 mM Tris, 5% Sucrose, 1% (10 mg/mL) rHSA, pH 7.4 ± 0.2). The final target volume of DS should be in the range of approximately 2 L. Based on current data the estimated range of the dilution factor might be 1:20 to 1:50.

Final DS sterile filtration

The final DS was filtered under aseptic conditions in a laminar flow hood using a sterility grade 0.2 µm syringe filter (e.g. 0.2 µm Mini Kleenpak EKV filter capsule with 220 cm^2 filter surface, Pall).

Quantification of host cell DNA (hcDNA) host cell protein (HCP) and endotoxin

The residual host cell DNA content of the sucrose gradient pool samples was determined by using the qPCR based assay. The DNA content in SGC pool was determined to be $\leq$ 0.002 ng/mL.The presence of residual host cell protein (HCP) from Vero cells was determined by ELISA. Residual host cell proteins present in the sucrose gradient pool samples were quantified using the Vero Cell HCP ELISA kit (Cygnus, F500). The residual host cell protein content in SGC pool was determined to be $\leq$ 200 ng/mL.

Endotoxin content of the SGC pool and DS was measured by Endosafe®-PTSTM system (Charles River). The system uses LAL reagents by a kinetic chromogenic methodology to measure color intensity directly related to the endotoxin content in a sample. Each cartridge contains precise amounts of a licensed LAL reagent, chromogenic substrate and an endotoxin control standard. Samples were diluted 1:100 in WFI. The SGC Pool F7-F11 was determined to be < 5.00 EU/mL; likewise, the Drug Substance was also determined to have < 5.00 EU/mL.

The following specifications for impurities in final Drug product were proposed: hcDNA < 10 ng/dose; Endotoxins < 50 EU/dose; HCP < 200 ng/dose. These residual specifications would already be met in the highly concentrated SGC pool (~10 log TCID50/mL), which provides a high margin of safety considering the high dilution factor of SGC pool to final DP of > 1:1000.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of claim A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of claim A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 1-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 1-13 and able to pack a virulent Zika virus.

A4. The vaccine of any one of claims A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of claims A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of claim A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of claim A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of claim A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of claims A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of claims A1-A9, further comprising an adjuvant.

A11. The vaccine of claim A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of claim A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of claim A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 70) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 71).

A15. The vaccine of any one of claims A1-A14, further comprising one or more pharmaceutically acceptable excipient.

B1. A kit comprising a Zika virus vaccine of any one of claims A1-A15.

B2. The kit of claim B1, further comprising a second vaccine.

B3. The kit of claim B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising

administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of claims A1-A15 to a subject in need thereof.

C2. The method of claim C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of claim C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of claim C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of claim C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of claims C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of claim D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of claim D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of claims D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of claim D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of claims D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of claim D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of claim D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of claims D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of claim D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of claim D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of claims A1-A15 for the treatment and prevention of a Zika virus infection.

E2. The use of claim E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of claim E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of claim E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of claim E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of claim E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of claims E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment and prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of claims A1-A15.

F2. The pharmaceutical composition of claim F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of claim F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of claim F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of claim F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of claim F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of claims F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

CLAIMS

What is claimed is:

1. A process of purification of infectious virus particles, comprising the steps of:
(a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;
(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);
(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

2. The process of claim 2, wherein the virus particles are selected from the group consisting of flaviviruses, e.g. yellow fever virus or Zika virus and alphaviruses, e.g. Chikungunya.

3. The process of claim 1 or claim 2, additionally comprising the step of:
(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

4. The process of any of claims 1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

5. The process of any of claims 1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

6. The process of claim 5, wherein the one or more pre-purification step(s) comprises
(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or
(b) digestion of host cell genomic DNA by enzymatic treatment; and/or
(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

7. The process of any one of claims 1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

8. The process of any one of claims 1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

9. The process of any one of claims 5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of claims 5 to 8 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

10. The process of any one of claims 1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

11. The process of any one of claims 1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

12. The process of claim 11, wherein said cell line is a Vero cell line.

13. The process of any one of claims 1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

14. The process of any one of claims 1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

15. The process of any one of claims 1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

16. Use of the process according to any one of claims 1 to 15 for manufacturing a composition for immunization against a virus infection.

17. The use according to claim 16, wherein the composition for immunization against a virus infection is an infection caused by a group of viruses consisting of yellow fever virus, Chikungunya virus and Zika virus.

18. A composition comprising the virus particles obtainable or obtained by the process of any one of claims 1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

20. The Zika virus vaccine of claim 19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

21. The vaccine of claim 19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

22. The vaccine of any one of claims 19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

23. The vaccine of any one of claims 19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

24. The vaccine of claim 23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

25. The vaccine of claim 24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

26. The vaccine of any one of claims 19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

27. The vaccine of claim 26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

28. The vaccine of claim 27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

29. The vaccine of claim 28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

30. The vaccine of any one of claims 27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

31. The vaccine of any one of claims 19 to 30, further comprising an adjuvant.

32. The vaccine of claim 31, wherein the adjuvant is an aluminum salt adjuvant.

33. The vaccine of claim 32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

34. The vaccine of claim 32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

35. The vaccine of any one of claims 19 to 34, further comprising one or more pharmaceutically acceptable excipient.

ABSTRACT

Described herein are improved purification methods for virus vaccines and compositions. Also described are Zika vaccines and methods of producing and administering said vaccines to subjects in need thereof.

Figure 1

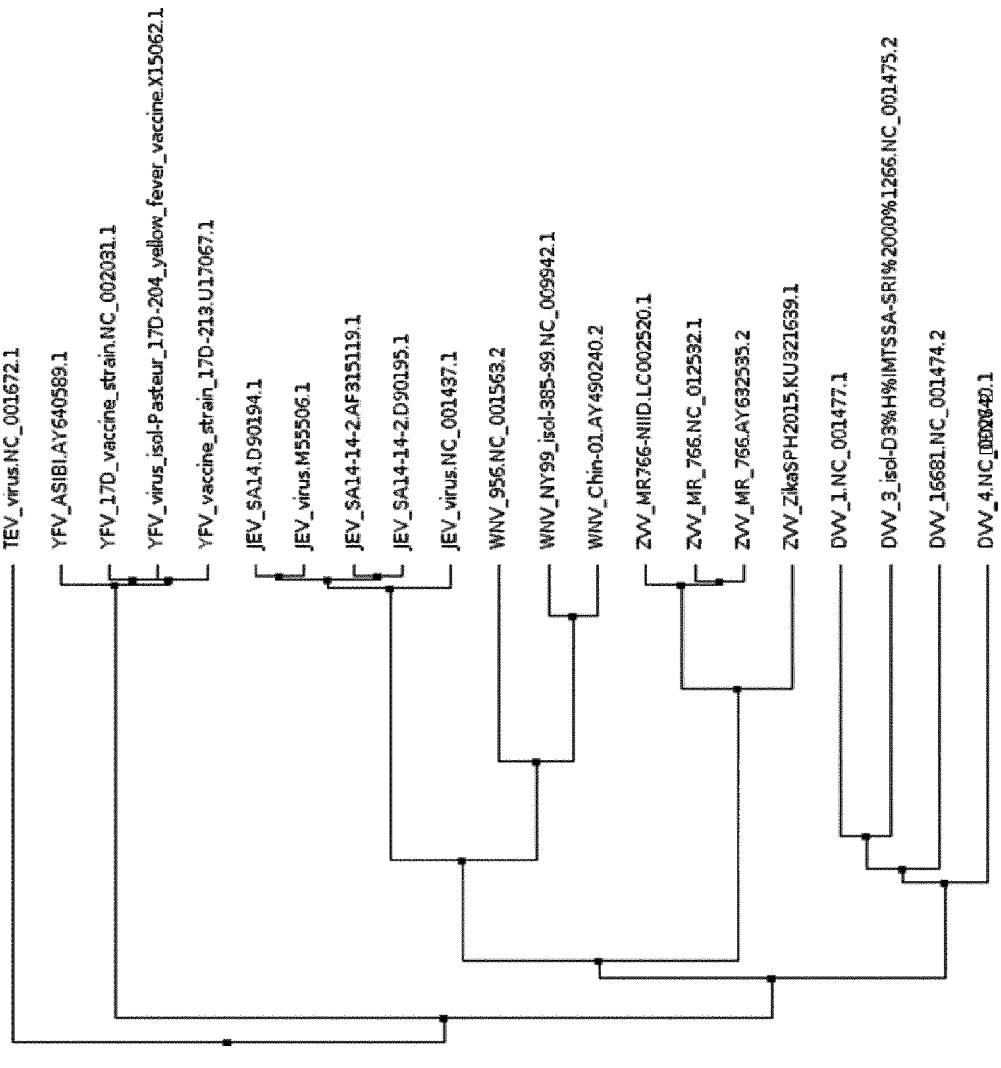

TEV_virus.NC_001672.1
YFV_ASIB1.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
ZW_MR766-NIID.LC002520.1
ZW_MR_766.NC_012532.1
ZW_MR_766.AY632535.2
ZW_ZikaSPH2015.KU321639.1
DW_1.NC_001477.1
DW_3_isol-D3%H%IMTSSA-SRI%2000%1266.NC_001475.2
DW_16681.NC_001474.2
DW_4.NC_[illegible]

Figure 2

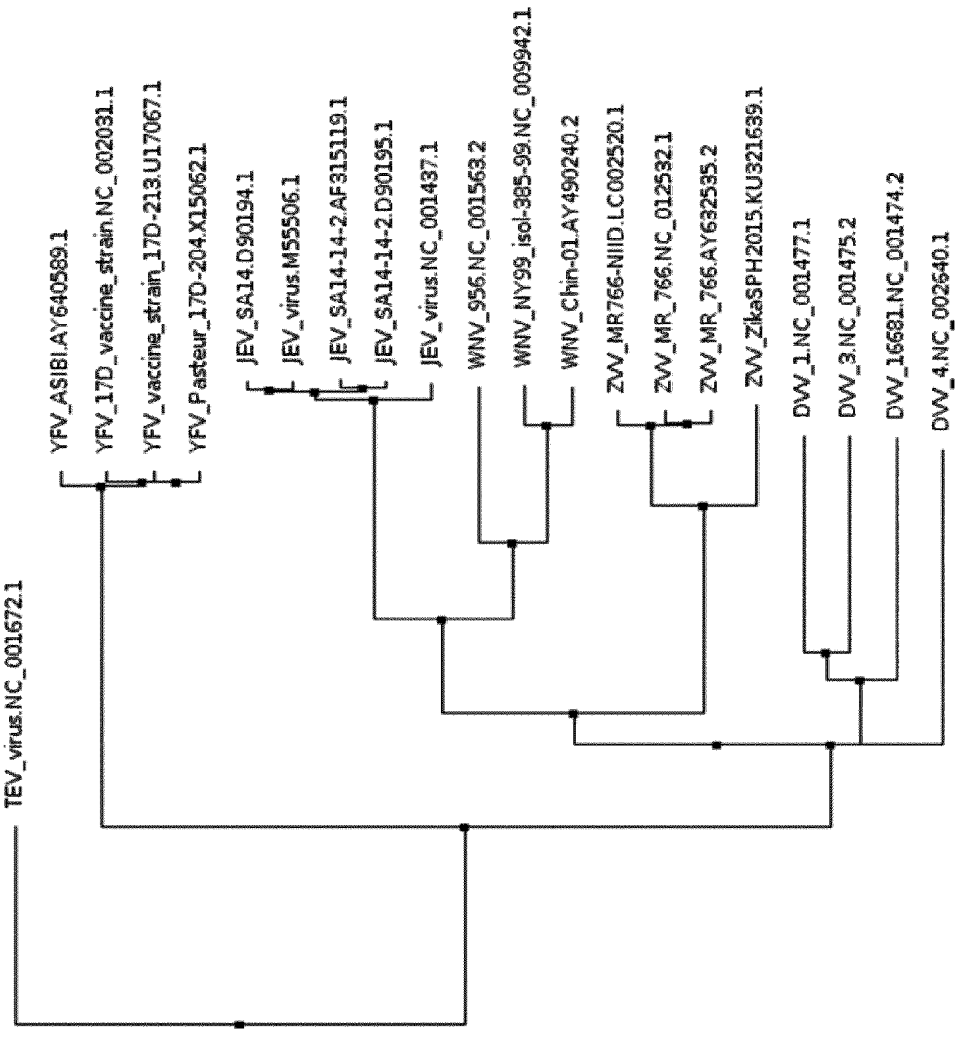

TEV_virus.NC_001672.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_vaccine_strain_17D-213.U17067.1
YFV_Pasteur_17D-204.X15062.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
ZW_MR766-NIID.LC002520.1
ZW_MR_766.NC_012532.1
ZW_MR_766.AY632535.2
ZW_ZikaSPH2015.KU321639.1
DW_1.NC_001477.1
DW_3.NC_001475.2
DW_16681.NC_001474.2
DW_4.NC_002640.1

Figure 3

		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DVV_1.NC_001477.1	**0**	100.0	70.1	73.0	67.8	60.5	60.4	60.5	60.5	60.7	55.8	60.6	60.3	60.6	58.0	57.9	57.9	58.0	62.3	62.3	62.3	61.3
DVV_16681.NC_001474.2	**1**	70.1	100.0	70.8	68.6	60.5	60.5	60.5	60.6	60.7	54.9	60.9	60.3	60.4	58.3	58.3	58.3	58.3	62.7	62.8	62.8	62.2
DVV_3.NC_001475.2	**2**	73.0	70.8	100.0	68.9	60.2	60.1	60.1	60.1	60.1	55.8	60.3	60.0	60.1	57.5	57.6	57.5	57.5	62.6	62.7	62.7	62.5
DVV_4.NC_002640.1	**3**	67.8	68.6	68.9	100.0	60.5	60.6	60.6	60.6	60.4	55.4	60.8	60.3	60.5	58.0	57.9	57.9	57.9	62.1	62.0	62.0	62.1
JEV_SA14-14-2.AF315119.1	**4**	60.5	60.5	60.2	60.5	100.0	99.8	99.4	99.5	98.1	56.5	70.6	70.3	70.3	57.9	58.0	58.0	58.0	61.9	61.8	61.8	61.4
JEV_SA14-14-2.D90195.1	**5**	60.4	60.5	60.1	60.6	99.8	100.0	99.4	99.5	98.2	56.6	70.6	70.4	70.3	57.9	58.0	57.9	58.0	61.9	61.8	61.8	61.5
JEV_SA14.D90194.1	**6**	60.5	60.5	60.1	60.6	99.4	99.4	100.0	99.8	98.6	56.6	70.8	70.5	70.4	58.0	58.0	58.0	58.0	61.9	61.8	61.8	61.6
JEV_virus.M55506.1	**7**	60.5	60.6	60.1	60.6	99.5	99.5	99.8	100.0	98.6	56.7	70.8	70.4	70.3	58.0	58.1	58.0	58.1	61.9	61.8	61.8	61.6
JEV_virus.NC_001437.1	**8**	60.7	60.7	60.1	60.4	98.1	98.2	98.6	98.6	100.0	56.6	70.6	70.5	70.6	58.3	58.3	58.4	58.3	62.1	62.0	62.0	61.7
TEV_virus.NC_001672.1	**9**	55.8	54.9	55.8	55.4	56.5	56.6	56.6	56.7	56.6	100.0	56.2	56.7	56.6	56.9	57.0	57.0	57.0	56.6	56.7	56.7	56.7
WNV_956.NC_001563.2	**10**	60.6	60.9	60.3	60.8	70.6	70.6	70.8	70.8	70.6	56.2	100.0	79.6	79.6	58.1	58.0	58.1	58.2	62.4	62.3	62.3	62.2
WNV_Chin-01.AY490240.2	**11**	60.3	60.3	60.0	60.3	70.3	70.4	70.5	70.4	70.5	56.7	79.6	100.0	95.3	58.2	58.2	58.2	58.2	62.0	62.1	62.1	62.7
WNV_NY99_isol-385-99.NC_009942.1	**12**	60.6	60.4	60.1	60.5	70.3	70.3	70.4	70.3	70.6	56.6	79.6	95.3	100.0	57.8	57.8	57.8	57.8	62.0	62.0	62.0	62.4
YFV_17D_vaccine_strain.NC_002031.1	**13**	58.0	58.3	57.5	58.0	57.9	57.9	58.0	58.0	58.3	56.9	58.1	58.2	57.8	100.0	99.4	99.9	99.9	58.5	58.5	58.5	58.2
YFV_ASIBI.AY640589.1	**14**	57.9	58.3	57.6	57.9	58.0	58.0	58.0	58.1	58.3	57.0	58.0	58.2	57.8	99.4	100.0	99.4	99.4	58.4	58.4	58.4	58.1
YFV_Pasteur_17D-204.X15062.1	**15**	57.9	58.3	57.5	57.9	58.0	57.9	58.0	58.0	58.4	57.0	58.1	58.2	57.8	99.9	99.4	100.0	99.9	58.4	58.4	58.4	58.2
YFV_vaccine_strain_17D-213.U17067.1	**16**	58.0	58.3	57.5	57.9	58.0	58.0	58.0	58.1	58.3	57.0	58.2	58.2	57.8	99.9	99.4	99.9	100.0	58.4	58.4	58.4	58.1
ZVV_MR766-NIID.LC002520.1	**17**	62.3	62.7	62.6	62.1	61.9	61.9	61.9	61.9	62.1	56.6	62.4	62.0	62.0	58.5	58.4	58.4	58.4	100.0	99.7	99.7	88.9
ZVV_MR_766.AY632535.2	**18**	62.3	62.8	62.7	62.0	61.8	61.8	61.8	61.8	62.0	56.7	62.3	62.1	62.0	58.5	58.4	58.4	58.4	99.7	100.0	100.0	88.8
ZVV_MR_766.NC_012532.1	**19**	62.3	62.8	62.7	62.0	61.8	61.8	61.8	61.8	62.0	56.7	62.3	62.1	62.0	58.5	58.4	58.4	58.4	99.7	100.0	100.0	88.8
ZVV_ZikaSPH2015.KU321639.1	**20**	61.3	62.2	62.5	62.1	61.4	61.5	61.6	61.6	61.7	56.7	62.2	62.7	62.4	58.2	58.1	58.2	58.1	88.9	88.8	88.8	100.0

60 % Identity and higher
70 % Identity and higher
80 % Identity and higher

Figure 4

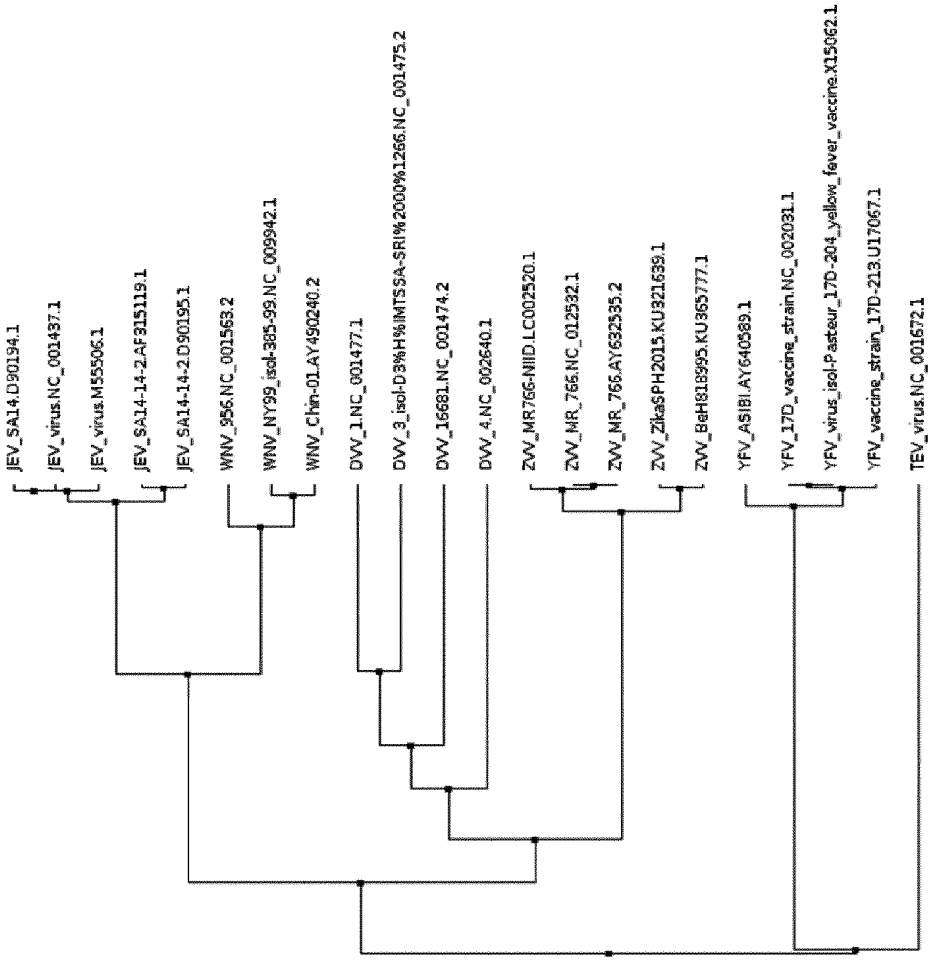

JEV_SA14.D90194.1
JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
DVV_1.NC_001477.1
DVV_3_isol-D3%H%IMTSA-SRI%2000%1266.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
ZVV_BeH818995.KU365777.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1

Figure 5

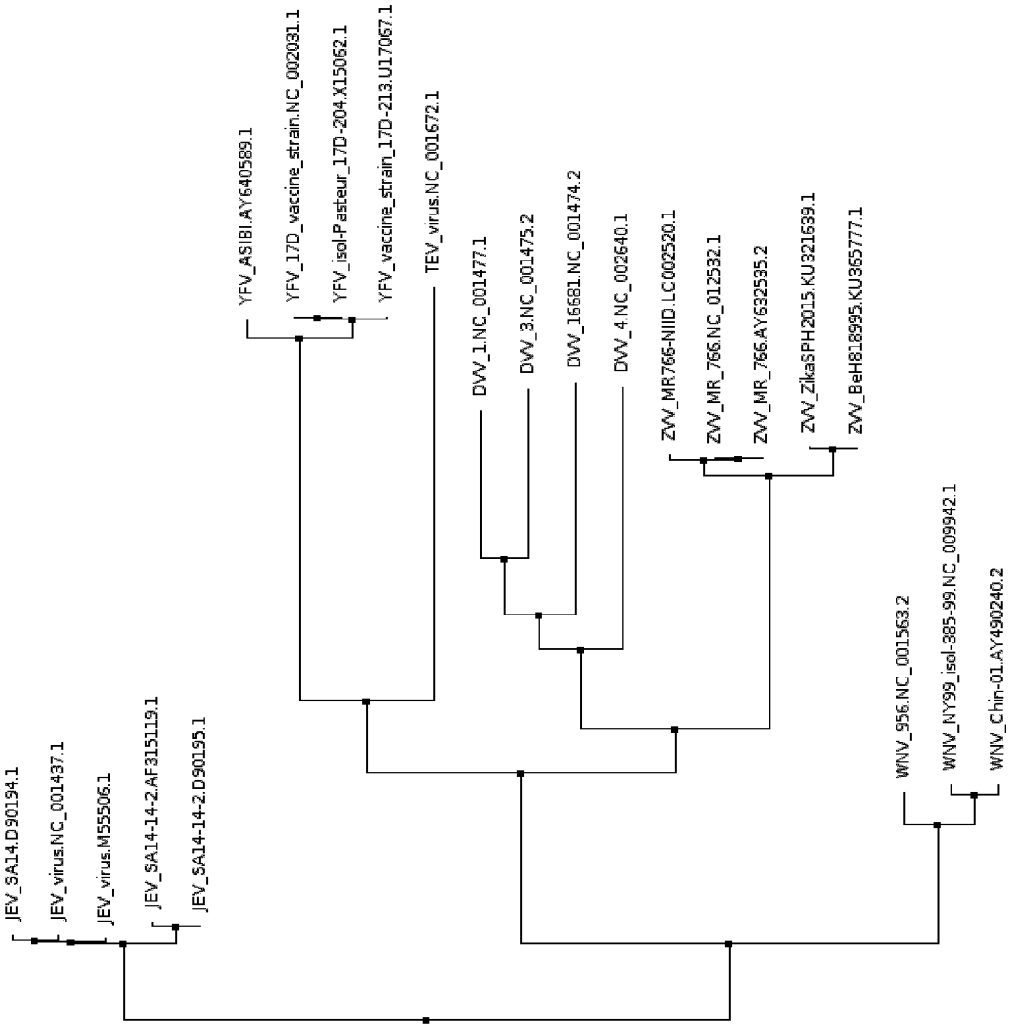

JEV_SA14.D90194.1
JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_isol-Pasteur_17D-204.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1
DVV_1.NC_001477.1
DVV_3.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
ZVV_BeH818995.KU365777.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2

Figure 6

		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
DVV_1.NC_001477.1	0	100.0	68.7	77.4	63.8	49.7	49.7	50.1	49.9	50.5	38.3	51.5	50.5	50.9	43.0	43.8	43.0	43.2	57.3	57.9	58.4	58.4	57.1
DVV_16681.NC_001474.2	1	68.7	100.0	68.7	64.0	46.7	46.7	47.1	46.9	47.5	38.9	48.4	47.6	47.6	43.6	44.4	43.6	43.6	53.6	53.8	54.2	54.2	53.8
DVV_3.NC_001475.2	2	77.4	68.7	100.0	62.8	48.0	48.0	48.2	48.2	48.6	38.2	46.8	46.7	46.3	41.6	42.2	41.6	41.7	57.3	57.7	58.2	58.2	57.1
DVV_4.NC_002640.1	3	63.8	64.0	62.8	100.0	47.5	47.5	47.7	47.5	48.1	39.7	49.6	49.6	49.8	40.0	40.6	40.0	40.0	55.8	56.4	56.4	56.4	55.8
JEV_SA14-14-2.AF315119.1	4	49.7	46.7	48.0	47.5	100.0	99.8	98.0	98.6	97.8	39.6	76.1	76.5	76.7	43.1	42.9	43.1	43.1	54.0	54.8	54.8	54.8	54.0
JEV_SA14-14-2.D90195.1	5	49.7	46.7	48.0	47.5	99.8	100.0	98.2	98.8	98.0	39.8	76.3	76.7	76.9	43.1	42.9	43.1	43.1	54.0	54.8	54.8	54.8	54.0
JEV_SA14.D90194.1	6	50.1	47.1	48.2	47.7	98.0	98.2	100.0	99.4	99.4	40.0	77.5	77.8	78.0	43.3	43.3	43.3	43.3	54.4	55.4	55.2	55.2	54.4
JEV_virus.M55506.1	7	49.9	46.9	48.2	47.5	98.6	98.8	99.4	100.0	99.2	40.0	77.3	77.6	77.8	43.3	43.3	43.3	43.3	54.2	55.2	55.0	55.0	54.2
JEV_virus.NC_001437.1	8	50.5	47.5	48.6	48.1	97.8	98.0	99.4	99.2	100.0	40.4	78.0	78.4	78.6	43.5	43.5	43.5	43.5	54.8	55.4	55.2	55.2	54.8
TEV_virus.NC_001672.1	9	38.3	38.9	38.2	39.7	39.6	39.8	40.0	40.0	40.4	100.0	41.3	41.6	41.0	42.3	41.9	42.3	42.3	39.9	39.9	39.6	39.6	39.9
WNV_956.NC_001563.2	10	51.5	48.4	46.8	49.6	76.1	76.3	77.5	77.3	78.0	41.3	100.0	94.6	94.0	43.8	43.6	43.8	43.8	55.0	55.0	54.8	54.8	55.0
WNV_Chin-01.AY490240.2	11	50.5	47.6	46.7	49.6	76.5	76.7	77.8	77.6	78.4	41.6	94.6	100.0	93.8	43.9	43.5	43.9	43.9	54.4	54.2	54.0	54.0	54.4
WNV_NY99_isol-385-99.NC_009942.1	12	50.9	47.6	46.3	49.8	76.7	76.9	78.0	77.8	78.6	41.0	94.0	93.8	100.0	43.9	43.5	43.9	43.9	54.0	53.8	53.8	53.8	54.0
YFV_17D_vaccine_strain.NC_002031.1	13	43.0	43.6	41.6	40.0	43.1	43.1	43.3	43.3	43.5	42.3	43.8	43.9	43.9	100.0	97.6	100.0	99.8	42.0	43.4	43.5	43.5	42.0
YFV_ASIBI.AY640589.1	14	43.8	44.4	42.2	40.6	42.9	42.9	43.3	43.3	43.5	41.9	43.6	43.5	43.5	97.6	100.0	97.6	97.4	42.4	43.8	43.9	43.9	42.4
YFV_isol-Pasteur_17D-204.X15062.1	15	43.0	43.6	41.6	40.0	43.1	43.1	43.3	43.3	43.5	42.3	43.8	43.9	43.9	100.0	97.6	100.0	99.8	42.0	43.4	43.5	43.5	42.0
YFV_vaccine_strain_17D-213.U17067.1	16	43.2	43.6	41.7	40.0	43.1	43.1	43.3	43.3	43.5	42.3	43.8	43.9	43.9	99.8	97.4	99.8	100.0	42.0	43.4	43.5	43.5	42.0
ZVV_BeH818995.KU365777.1	17	57.3	53.6	57.3	55.8	54.0	54.0	54.4	54.2	54.8	39.9	55.0	54.4	54.0	42.0	42.4	42.0	42.0	100.0	96.8	96.4	96.4	99.8
ZVV_MR766-NIID.LC002520.1	18	57.9	53.8	57.7	56.4	54.8	54.8	55.4	55.2	55.4	39.9	55.0	54.2	53.8	43.4	43.8	43.4	43.4	96.8	100.0	98.8	98.8	96.6
ZVV_MR_766.AY632535.2	19	58.4	54.2	58.2	56.4	54.8	54.8	55.2	55.0	55.2	39.6	54.8	54.0	53.8	43.5	43.9	43.5	43.5	96.4	98.8	100.0	100.0	96.2
ZVV_MR_766.NC_012532.1	20	58.4	54.2	58.2	56.4	54.8	54.8	55.2	55.0	55.2	39.6	54.8	54.0	53.8	43.5	43.9	43.5	43.5	96.4	98.8	100.0	100.0	96.2
ZVV_ZikaSPH2015.KU321639.1	21	57.1	53.8	57.1	55.8	54.0	54.0	54.4	54.2	54.8	39.9	55.0	54.4	54.0	42.0	42.4	42.0	42.0	99.8	96.6	96.2	96.2	100.0

50 % Identity and higher
60 % Identity and higher
70 % Identity and higher

Figure 7 (a)

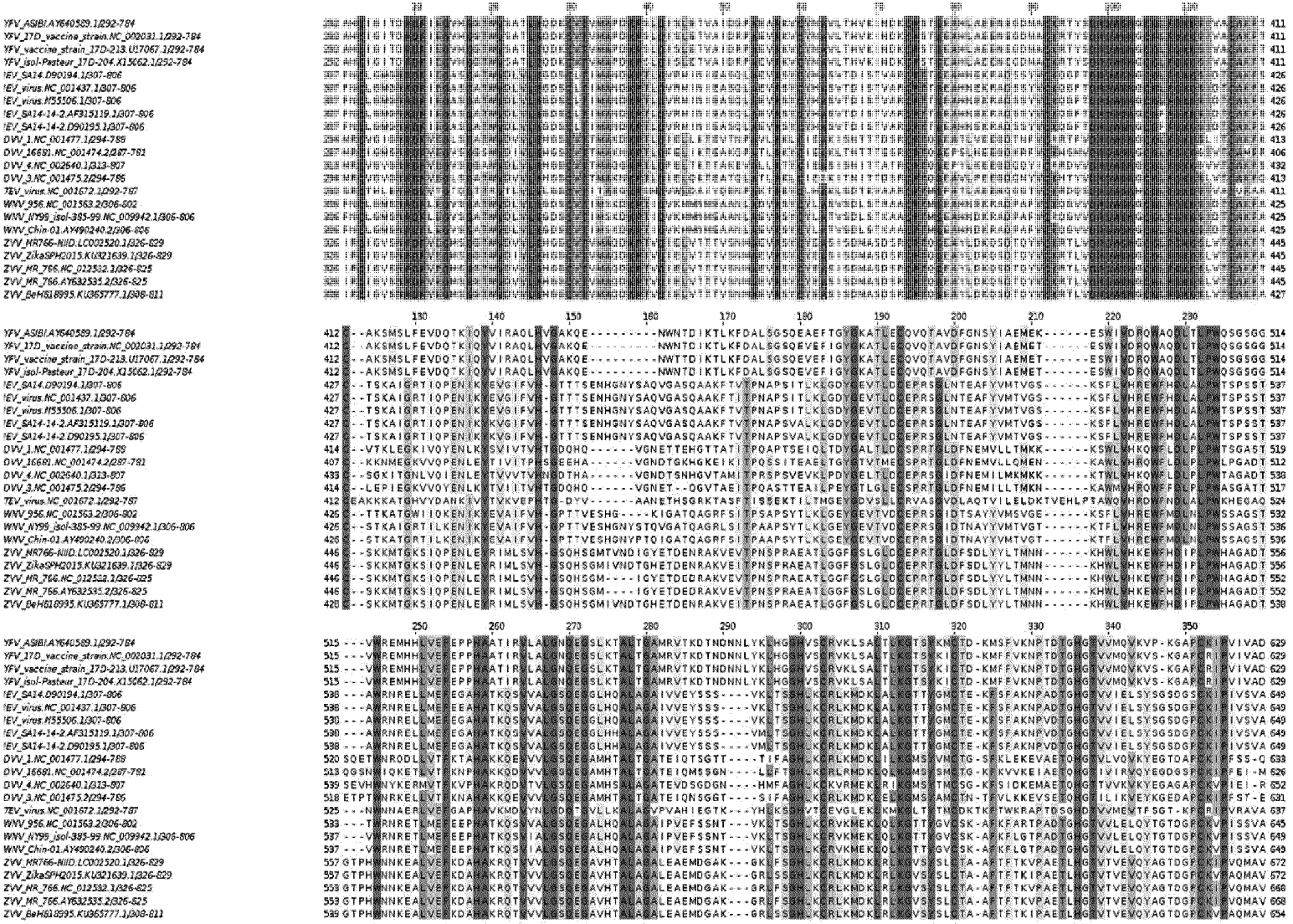

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DVV_4.NC_002640.1/313-807
DVV_3.NC_001475.2/294-786
TEV_virus.NC_001672.1/292-787
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WNV_Chin-01.AY490240.2/306-806
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Figure 7 (b)

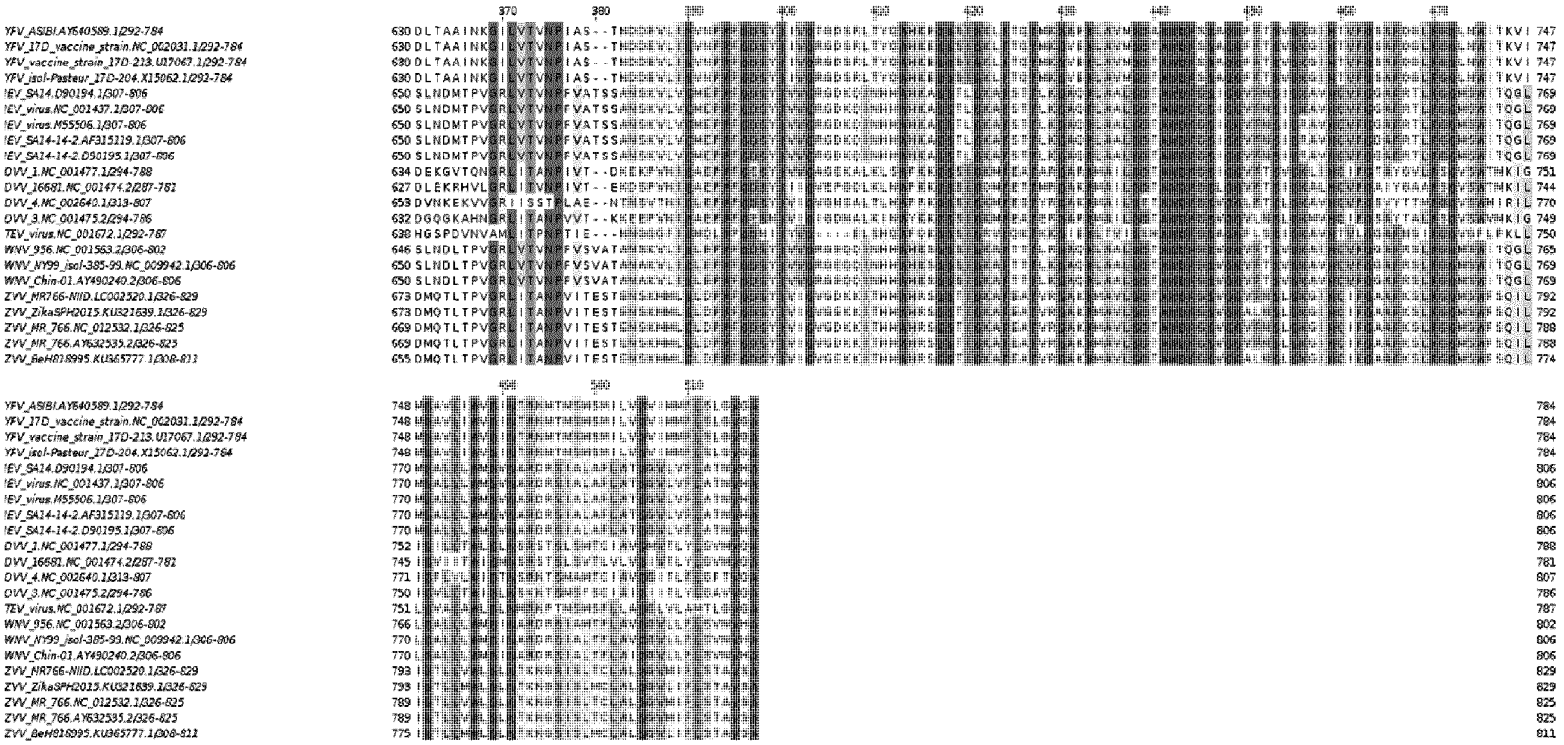

YFV_ASIB1.AY640589.1|292-784
YFV_17D_vaccine_strain.NC_002031.1|292-784
YFV_vaccine_strain_17D-213.U17067.1|292-784
YFV_isol-Pasteur_17D-204.X15062.1|292-784
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!EV_virus.NC_001437.1|307-806
!EV_virus.M55506.1|307-806
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!EV_SA14-14-2.D90195.1|307-806
DVV_1.NC_001477.1|294-788
DVV_16681.NC_001474.2|287-781
DVV_4.NC_002640.1|313-807
DVV_3.NC_001475.2|294-786
TEV_virus.NC_001672.1|292-787
WNV_956.NC_001563.2|306-802
WNV_NY99_isol-385-99.NC_009942.1|306-806
WNV_Chin-01.AY490240.2|306-806
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ZVV_ZikaSPH2015.KU321639.1|326-829
ZVV_MR_766.NC_012532.1|326-825
ZVV_MR_766.AY632535.2|326-825
ZVV_BeH818995.KU365777.1|308-811

Figure 8a

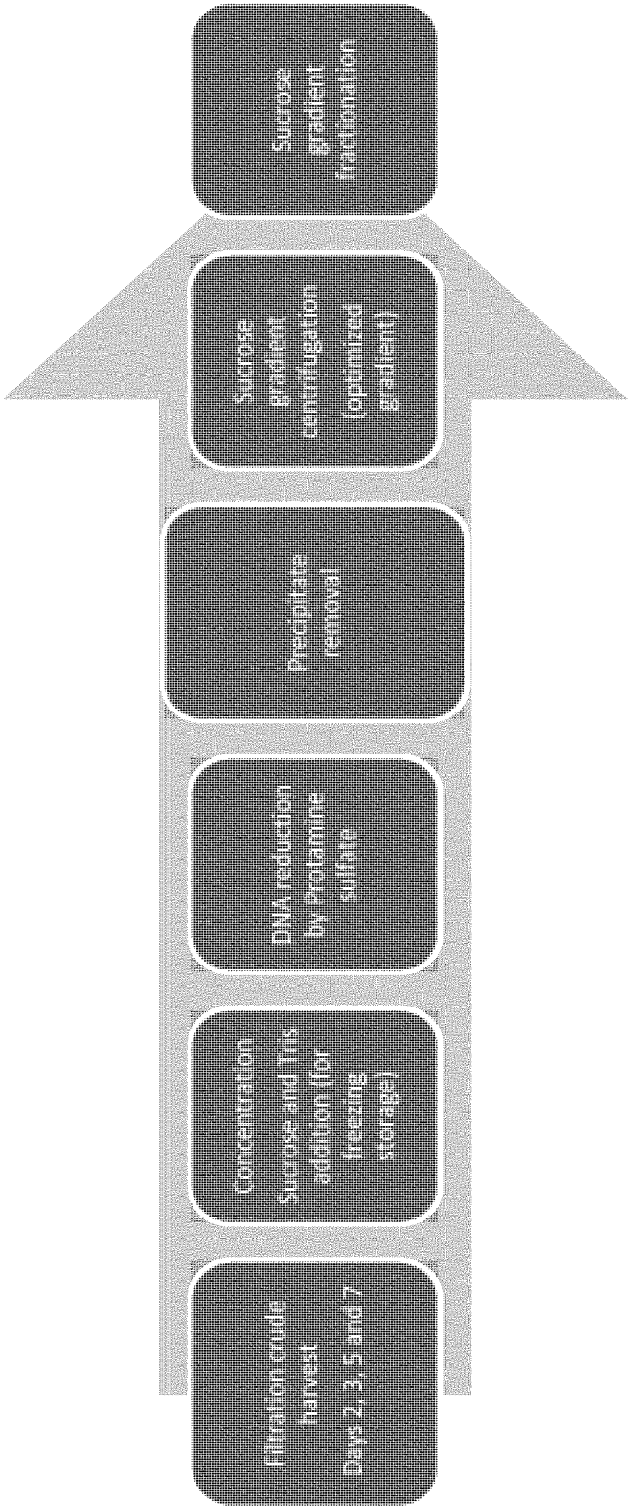

Immunomagnetic separation of target cells
Sucrose gradient centrifugation (optional step)
Preconcentrate (optional)
DNA restriction by endonuclease and/or shearing
Concentration and purification for amplification (optional step)
Immunomagnetic harvest
Days 2, 3, 5, and 7

Figure 8b

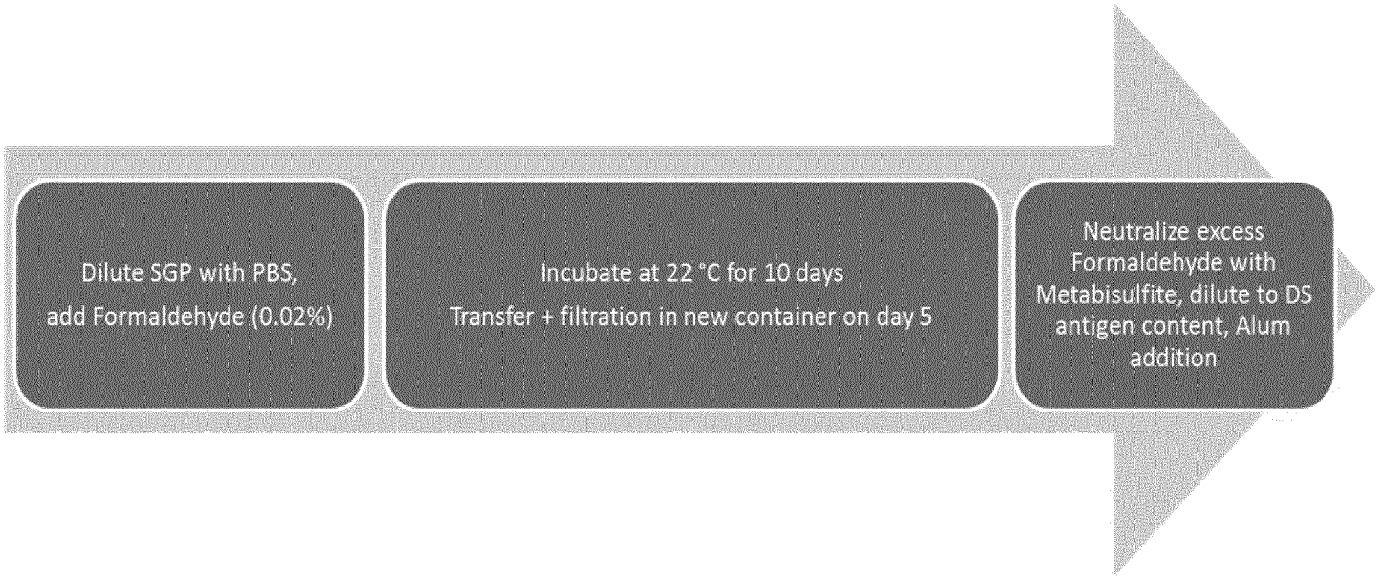
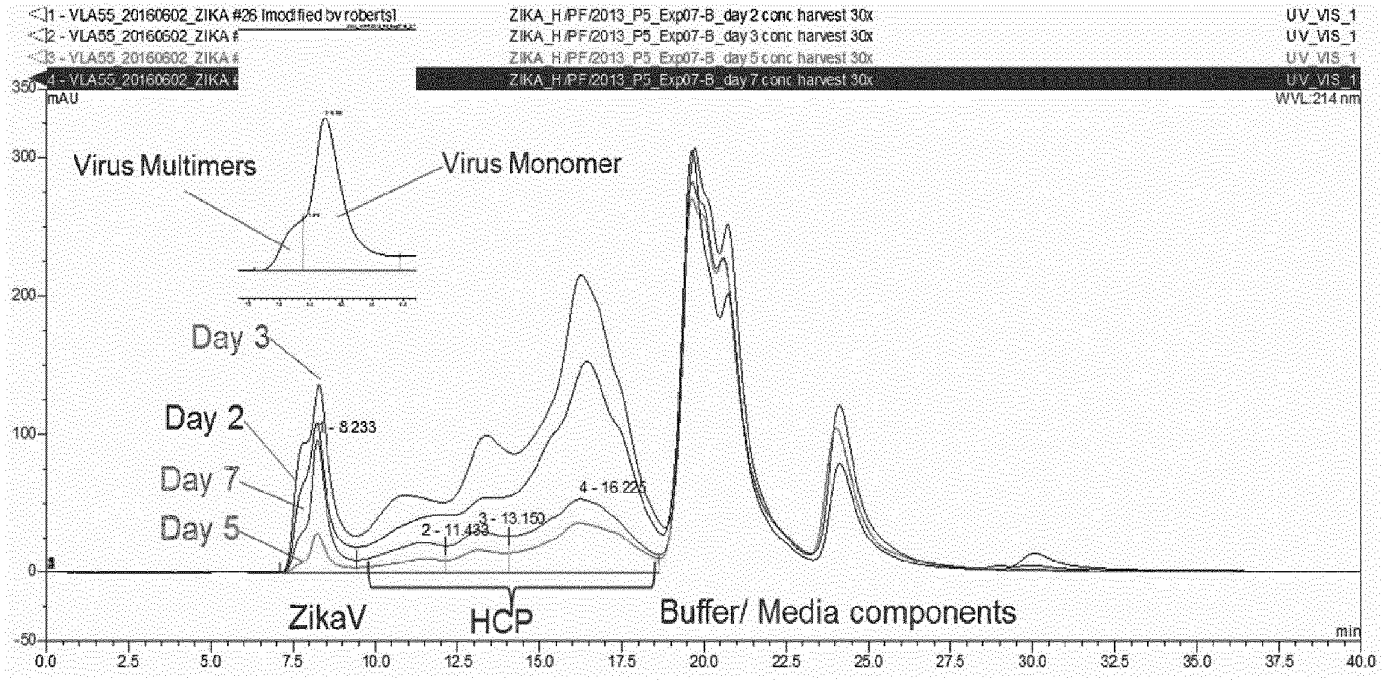
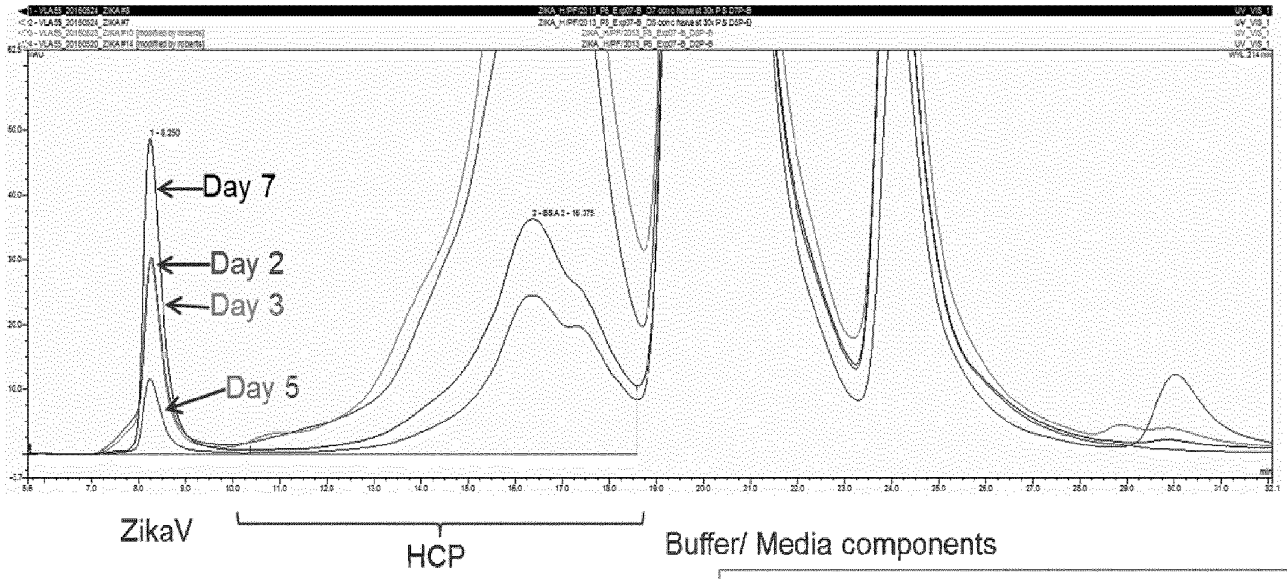

Dilute SGP with PBS,
add Formaldehyde (0.02%)
Incubate at 22 °C for 10 days
Transfer + filtration in new container on day 5
Neutralize excess Formaldehyde with Metabisulfite, dilute to DS antigen content, Alum addition

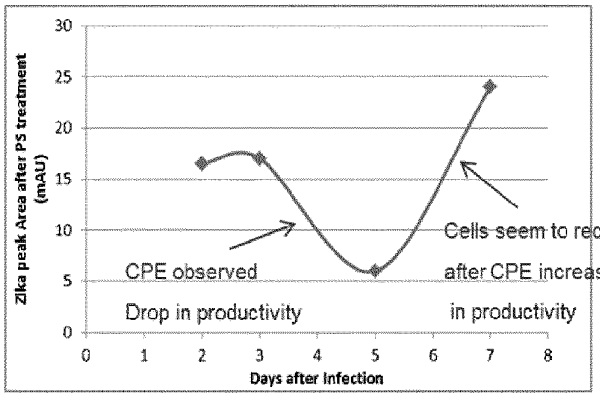
Figure 9



+ Similar virus concentration in harvests Day 2, Day 3, Day 7; lower virus concentration in harvest Day5
+ Day 2 and Day 3 harvests contain more HCP than Day 5 /7 (correlates with observed CPE)
+ Minor amount of virus multimers observed

Figure 10





+ Protamine Sulfate removes virus aggregates and partially HCP
+ Infectious virus titer is not affected by PS treatment
+ Strong CPE on day 3, drop in productivity
+ Remaining cells seem to recover well and increase in Zika productivity

Figure 11

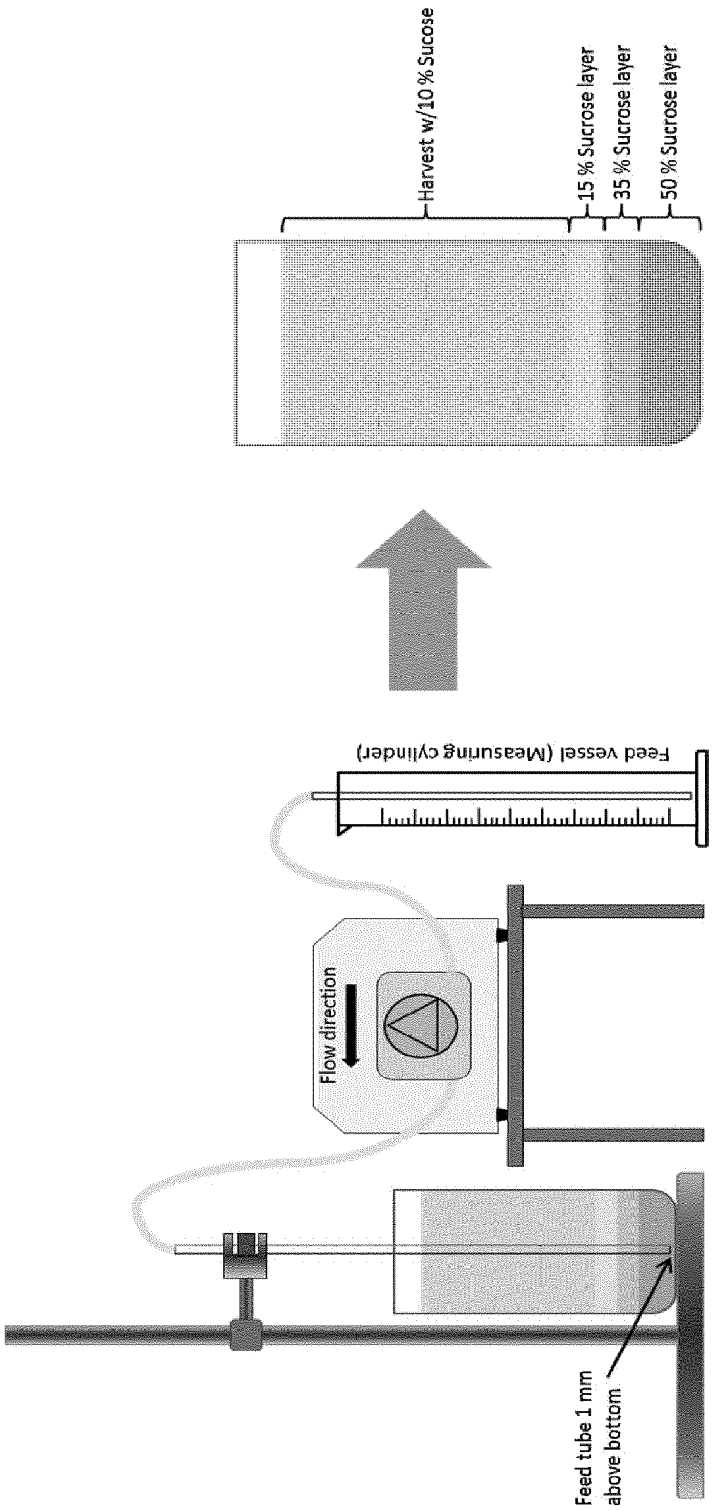
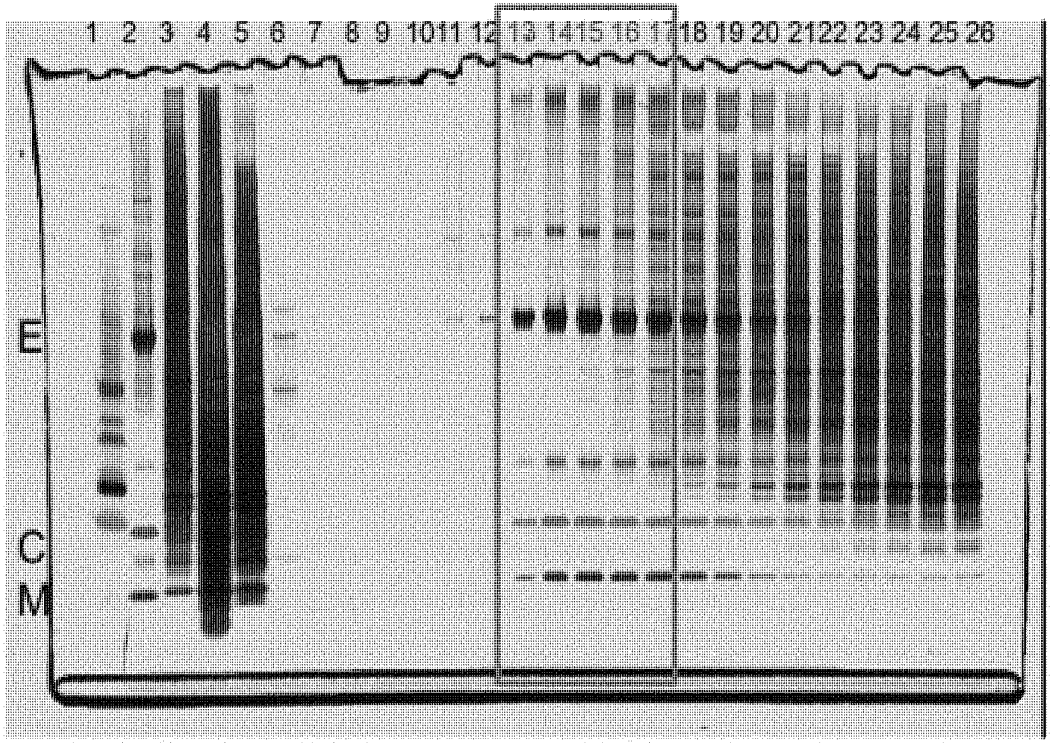

Harvest w/10 % Sucose
15 % Sucrose layer
35 % Sucrose layer
50 % Sucrose layer
Feed vessel (Measuring cylinder)
Flow direction
Feed tube 1 mm above bottom

Figure 12



Pooled fractions F10-F14
(10mL total volume per bottle)

Lane	Sample
1	Molecular weight Marker
2	JEV Sucrose Gradient Pool (Reference)
3	Zika Harvest pool (day 2,3,5,7)
4	Zika Harvest pool (day 2,3,5,7) concentrated 300x
5	Zika Harvest pool (day 2,3,5,7) concentrated 300x + Protamine sulfate
6	Ultrafiltration Permeate
7	SGC Bottle1, Fraction 4
8	SGC Bottle1, Fraction 5
9	SGC Bottle1, Fraction 6
10	SGC Bottle1, Fraction 7
11	SGC Bottle1, Fraction 8
12	SGC Bottle1, Fraction 9
13	SGC Bottle1, Fraction 10
14	SGC Bottle1, Fraction 11
15	SGC Bottle1, Fraction 12
16	SGC Bottle1, Fraction 13
17	SGC Bottle1, Fraction 14
18	SGC Bottle1, Fraction 15
19	SGC Bottle1, Fraction 16
20	SGC Bottle1, Fraction 17
21	SGC Bottle1, Fraction 18
22	SGC Bottle1, Fraction 19
23	SGC Bottle1, Fraction 20
24	SGC Bottle1, Fraction 21
25	SGC Bottle1, Fraction 22
26	SGC Bottle1, Fraction 23

Figure 13

Baseline separation of Virus and PS/HCP

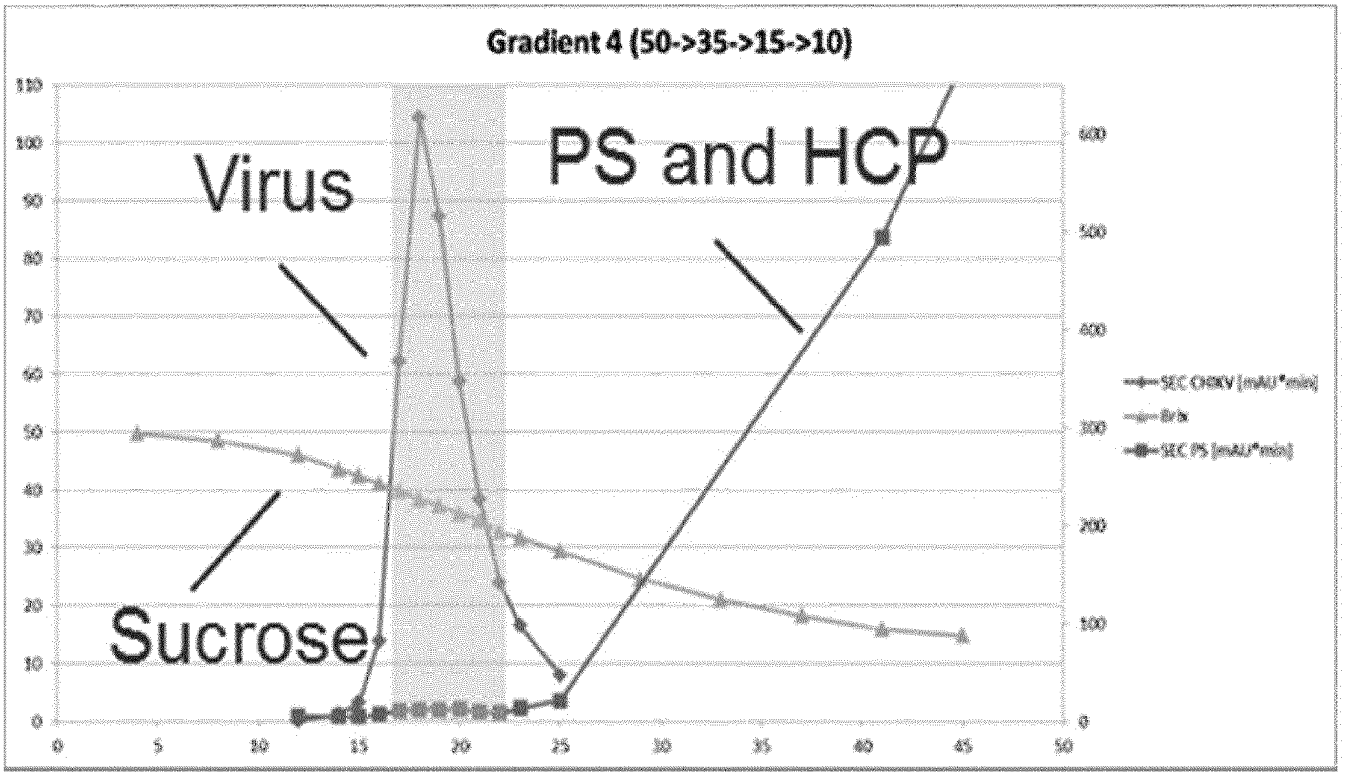


Figure 14

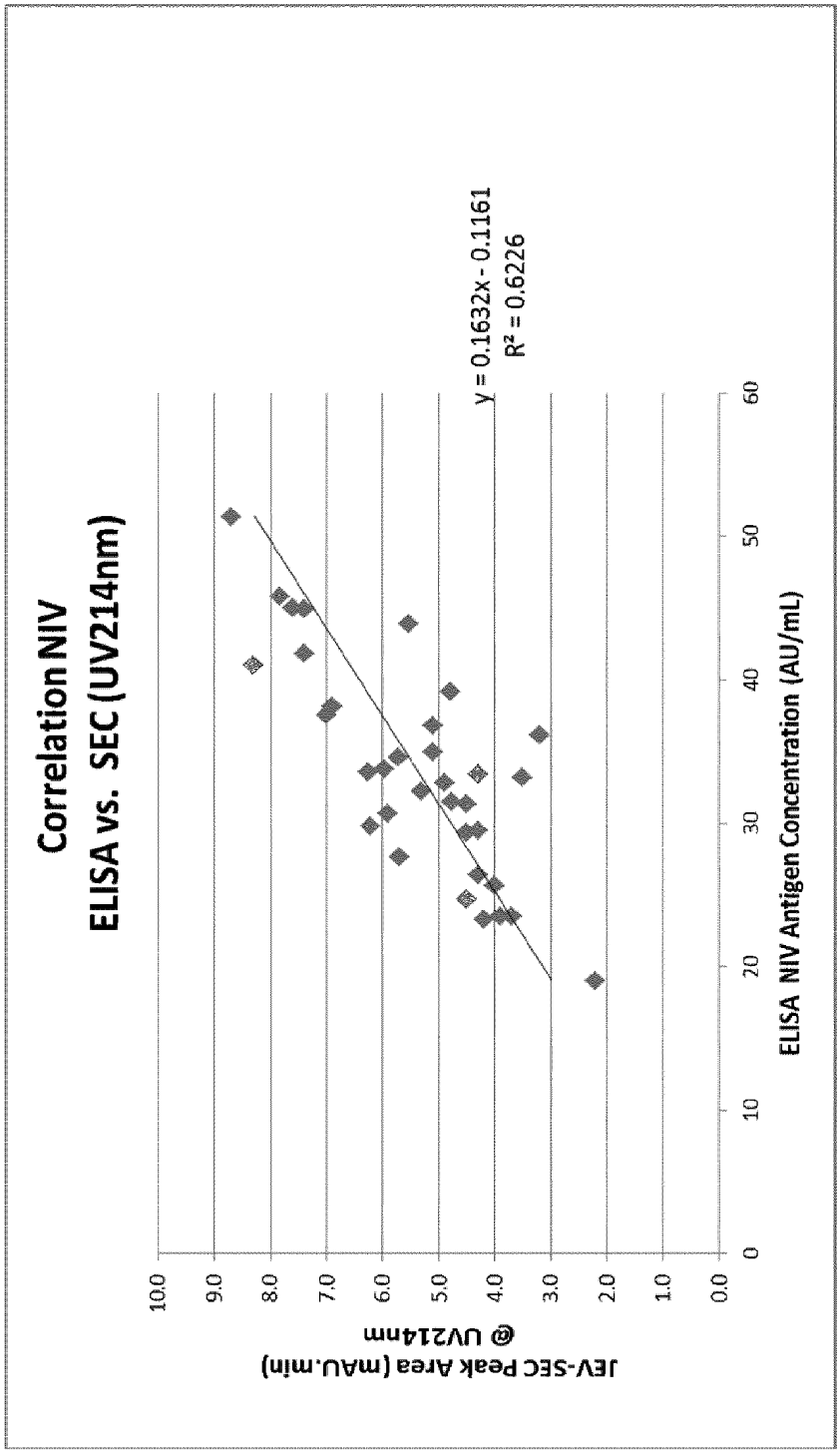
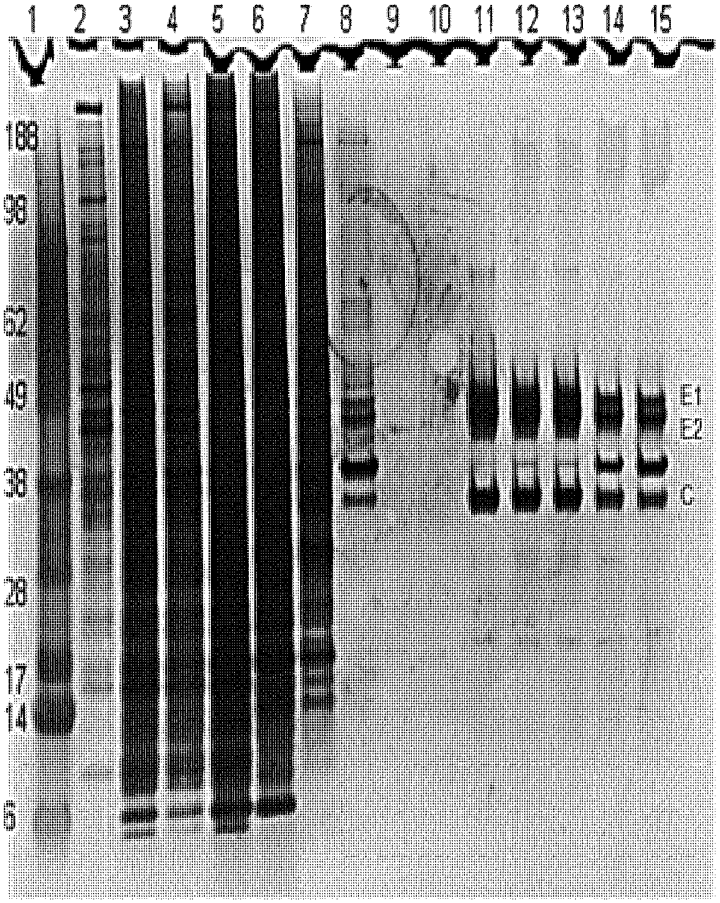

Correlation NIV
ELISA vs. SEC (UV214nm)
y = 0.1632x - 0.1161
R² = 0.6226
JEV-SEC Peak Area (mAU.min)
@ UV214nm
ELISA NIV Antigen Concentration (AU/mL)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
10
20
30
40
50
60

Figure 15



Lane	Sample
1	Marker Seeblueplus2
2	CHIKV lot20150812_WVB2015-01_Filtered harvest 0.2µ_24hpi
3	CHIKV lot20150812_WVB2015-01_Filtered harvest 0.2µ_48hpi
4	20150813_CHIKV_DSP_UF/DF_Load
5	20150813_CHIKV_DSP_UF/DF_conc. 10x
6	20150813_CHIKV_DSP_UF/DF_conc.&dia. 11x
7	20150813_CHIKV_DSP_PStreatment
8	20150813_CHIKV_DSP_PS&CC700treatment
9	20150813_CHIKV_DSP_SGCFrac F5
10	20150813_CHIKV_DSP_SGCFrac F6
11	20150813_CHIKV_DSP_SGCPool F7-F10
12	20150813_CHIKV_DSP_SGCPool F7-F11 (final pool)
13	20150813_CHIKV_DSP_SGCPool F7-F12
14	20150813_CHIKV_DSP_SGCFrac F13
15	20150813_CHIKV_DSP_SGCFrac F14

Figure 16a

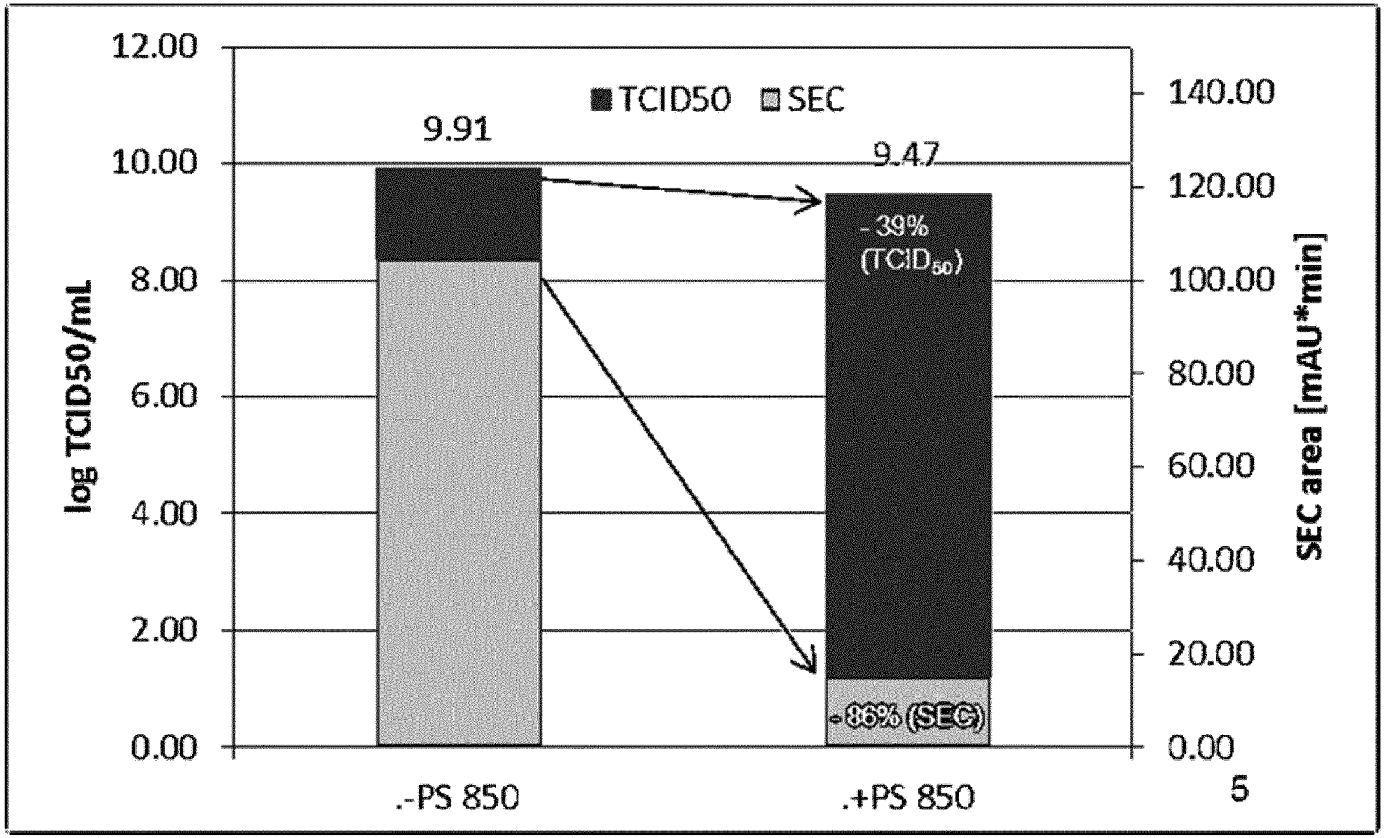
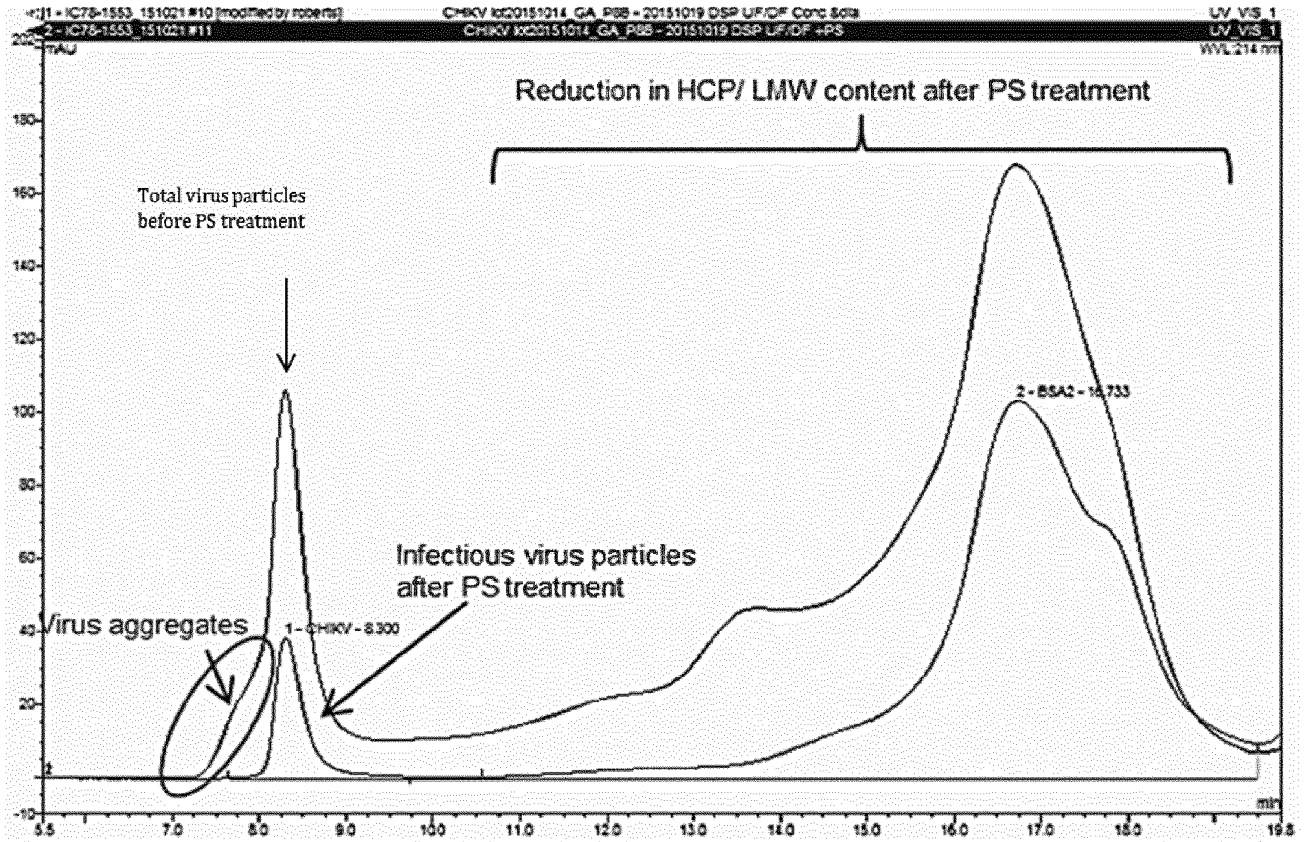

12.00
10.00
8.00
6.00
4.00
2.00
0.00
log TCID50/mL
TCID50
SEC
9.91
9.47
-39% (TCID50)
-86% (SEC)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
SEC area [mAU*min]
.-PS 850
.+PS 850
5

Figure 16b


Reduction in HCP/ LMW content after PS treatment
Total virus particles
before PS treatment
Infectious virus particles
after PS treatment
Virus aggregates
1 - CHIKV - 8.300
2 - BSA2 - 16.733
mAU
min

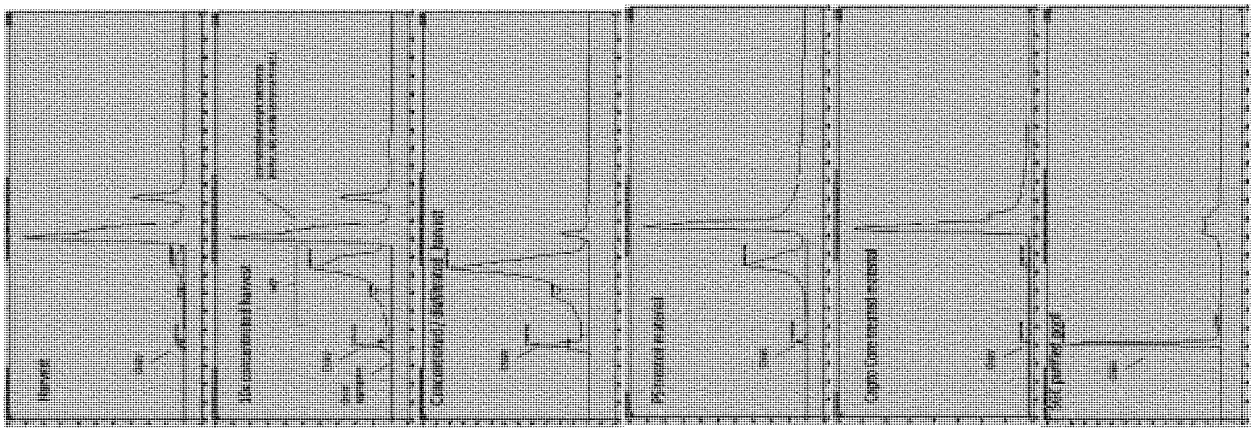


Figure 17

Figure 18a

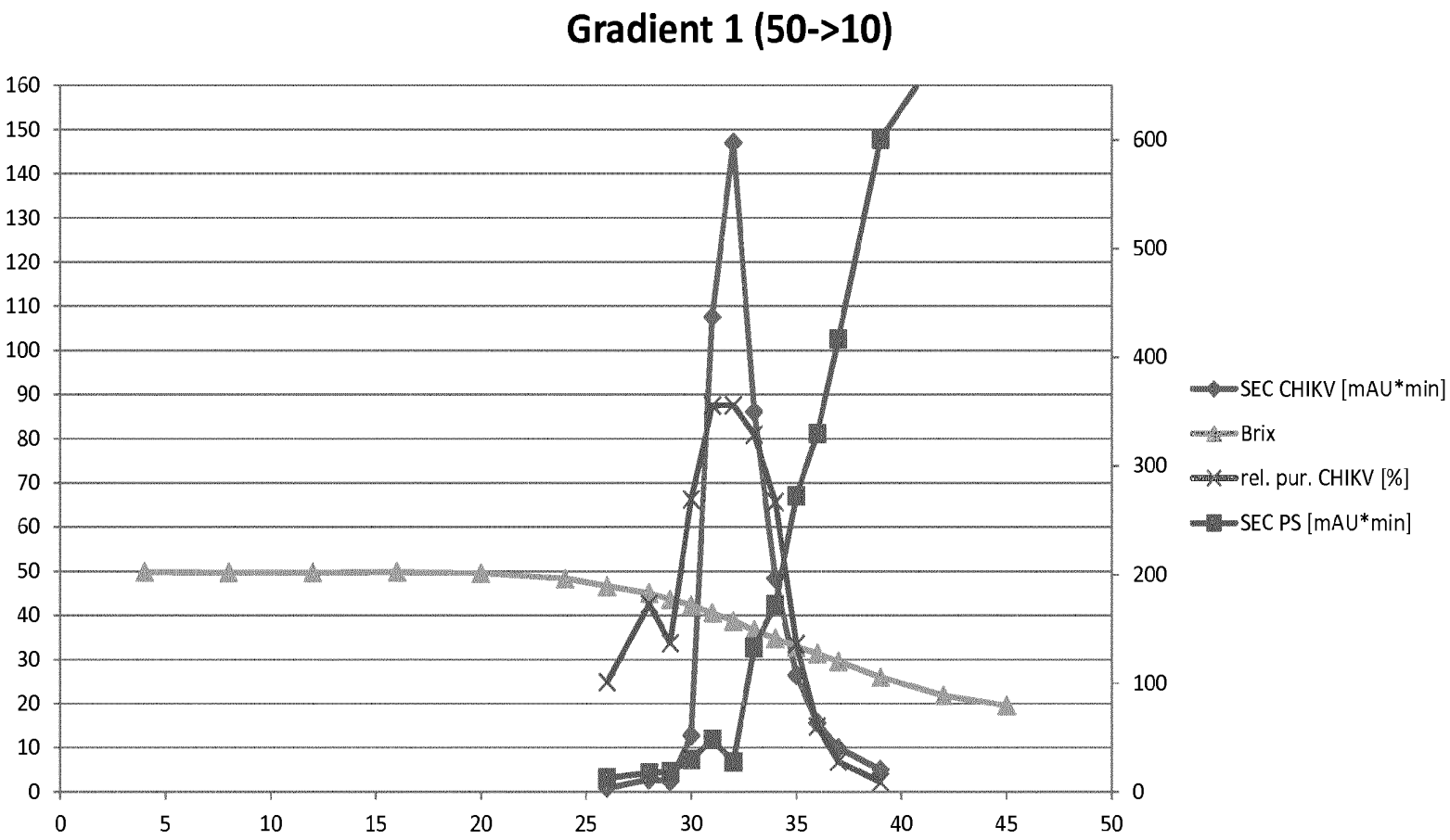

Gradient 1 (50->10)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
SEC CHIKV [mAU*min]
Brix
rel. pur. CHIKV [%]
SEC PS [mAU*min]

Figure 18b

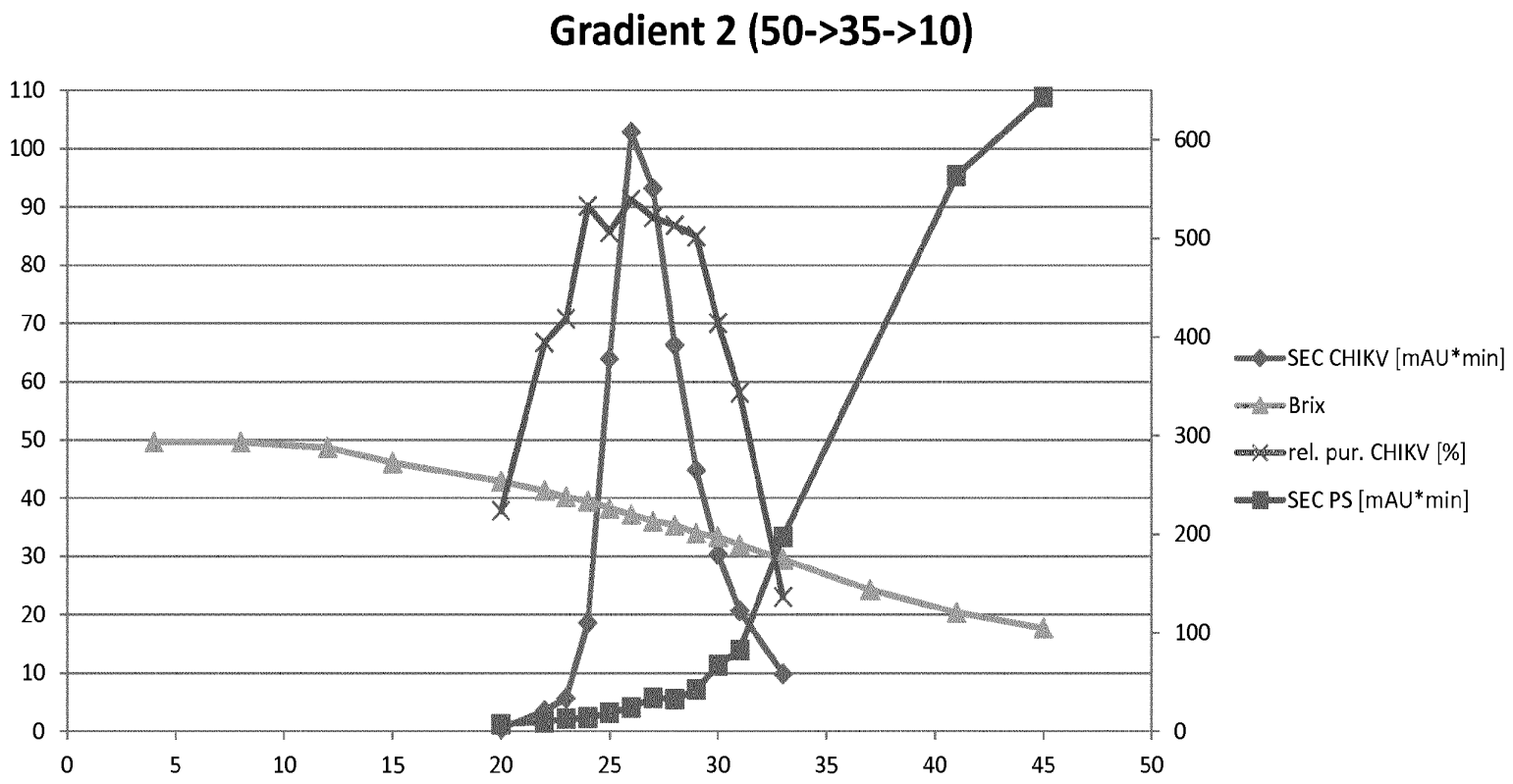

Gradient 2 (50->35->10)
110
100
90
80
70
60
50
40
30
20
10
0
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
SEC CHIKV [mAU*min]
Brix
rel. pur. CHIKV [%]
SEC PS [mAU*min]

Figure 18c

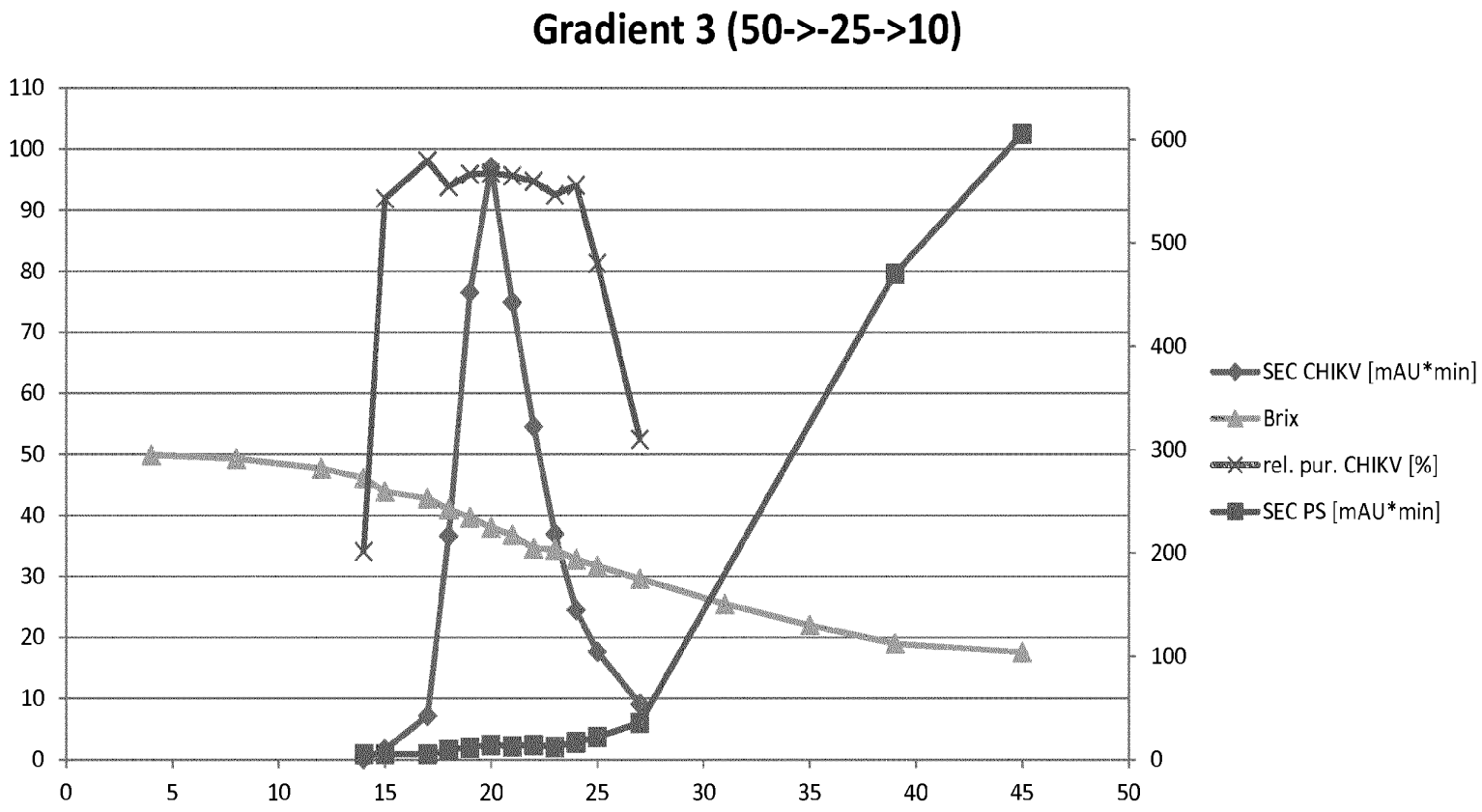

Gradient 3 (50->-25->10)
110
100
90
80
70
60
50
40
30
20
10
0
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
SEC CHIKV [mAU*min]
Brix
rel. pur. CHIKV [%]
SEC PS [mAU*min]

Figure 18d

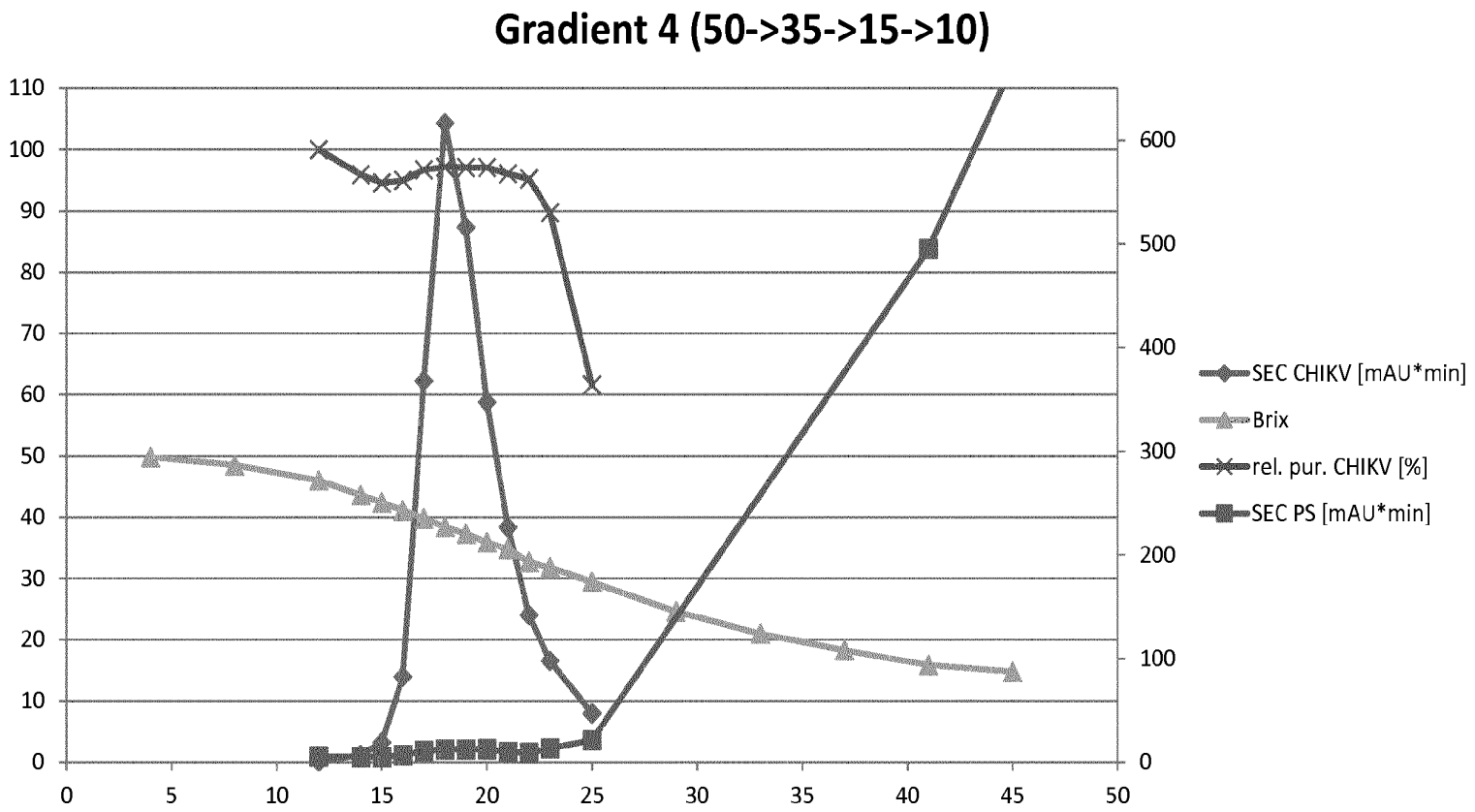

Gradient 4 (50->35->15->10)
110
100
90
80
70
60
50
40
30
20
10
0
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
SEC CHIKV [mAU*min]
Brix
rel. pur. CHIKV [%]
SEC PS [mAU*min]

Figure 19

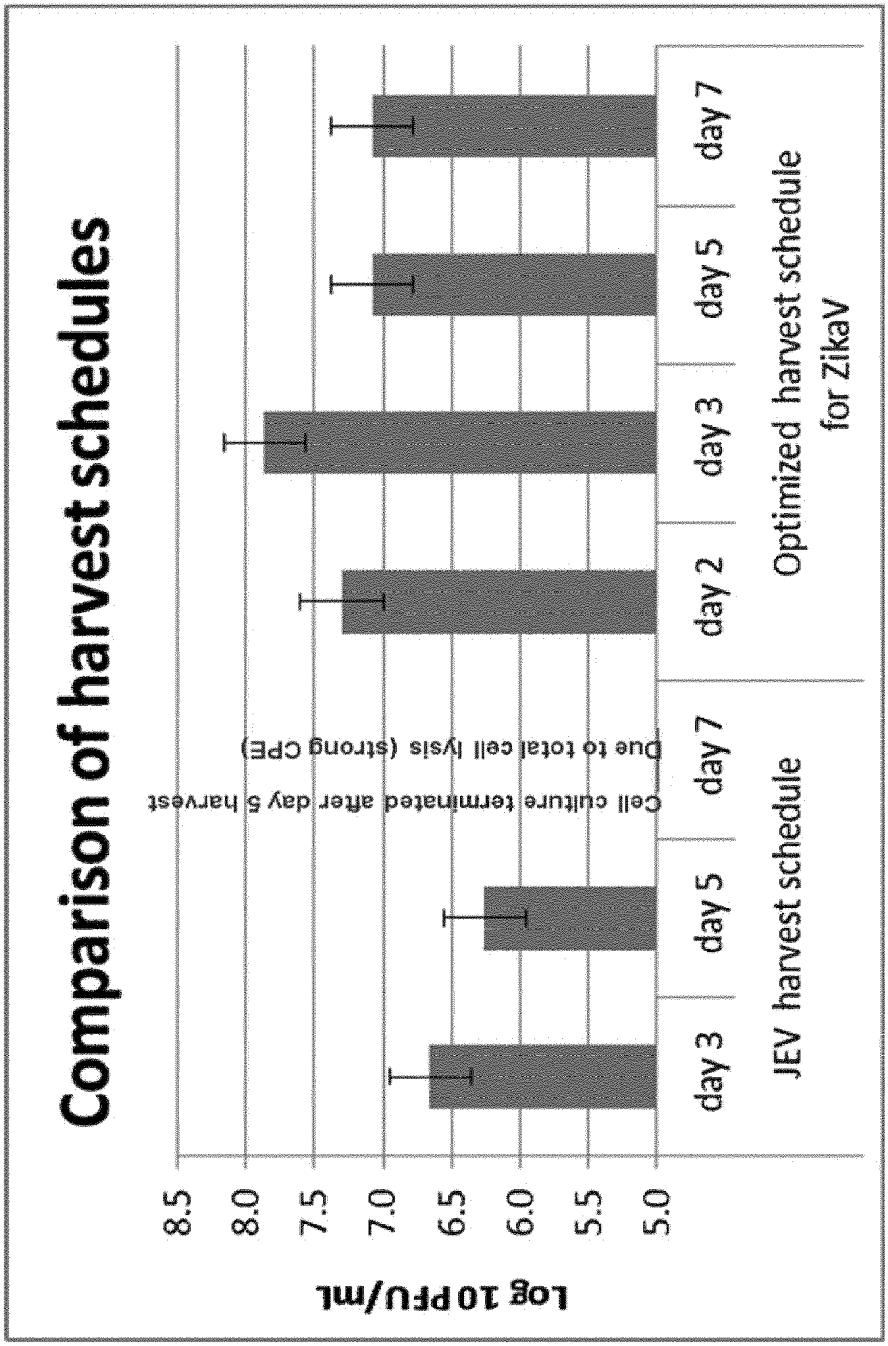
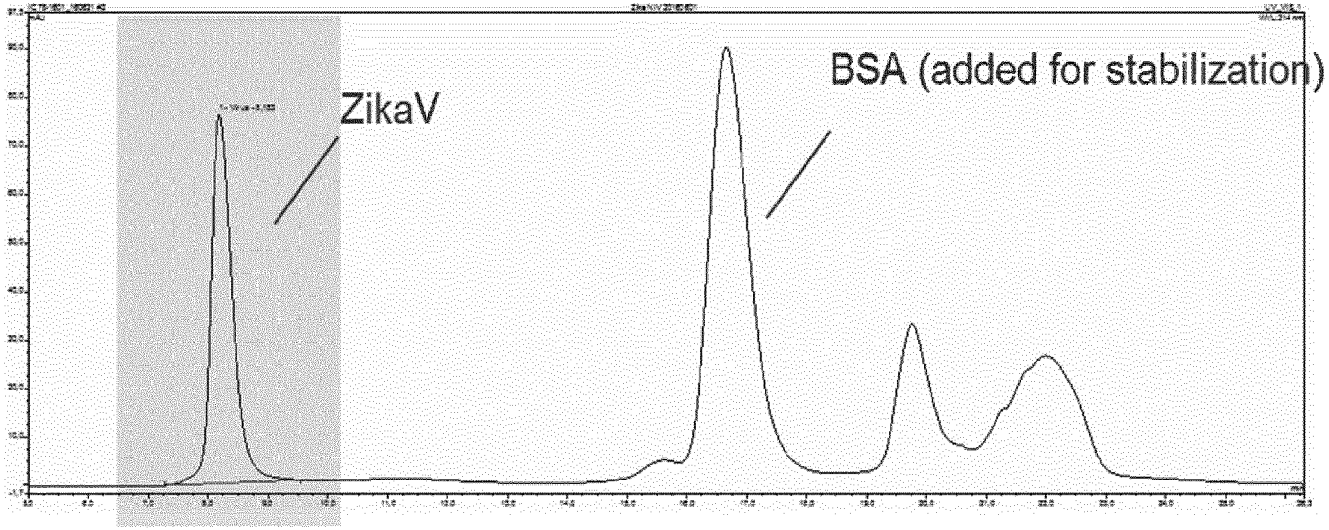

Comparison of harvest schedules
Log 10 PFU/mL
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
day 3
day 5
day 7
day 2
day 3
day 5
day 7
JEV harvest schedule
Optimized harvest schedule for ZikaV
Cell culture terminated after day 5 harvest
Due to total cell lysis (strong CPE)

Figure 20


ZikaV
BSA (added for stabilization)

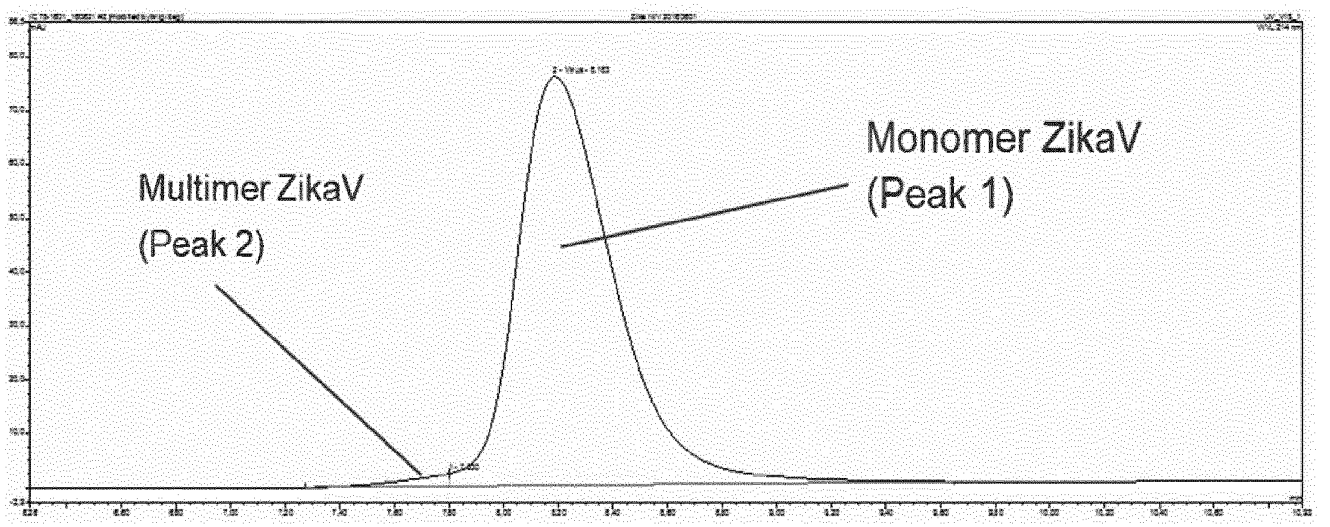

Multimer ZikaV
(Peak 2)
Monomer ZikaV
(Peak 1)

Figure 21

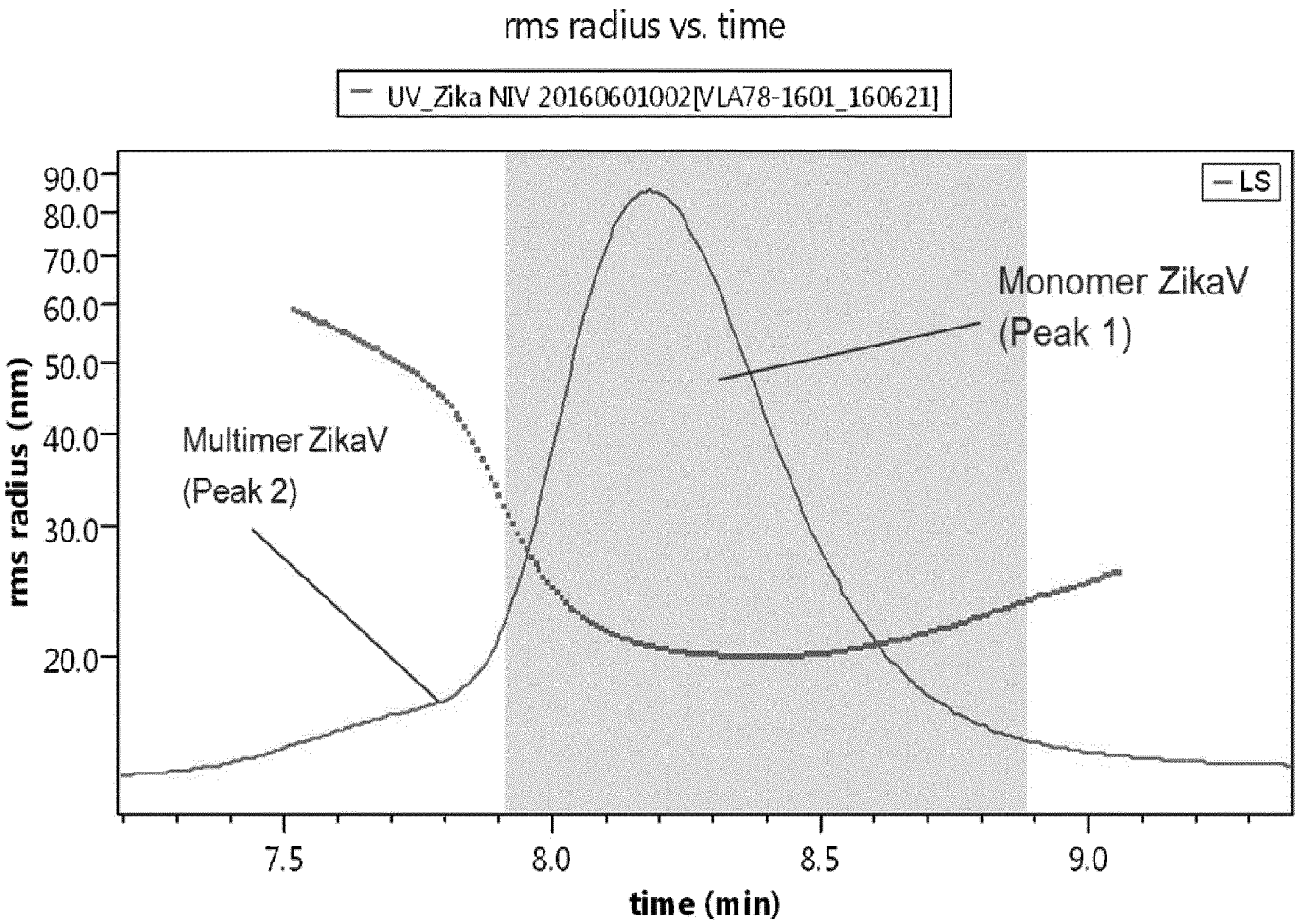

rms radius vs. time
UV_Zika NIV 20160601002[VLA78-1601_160621]
LS
Monomer ZikaV
(Peak 1)
Multimer ZikaV
(Peak 2)
rms radius (nm)
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
7.5
8.0
8.5
9.0
time (min)

Figure 22

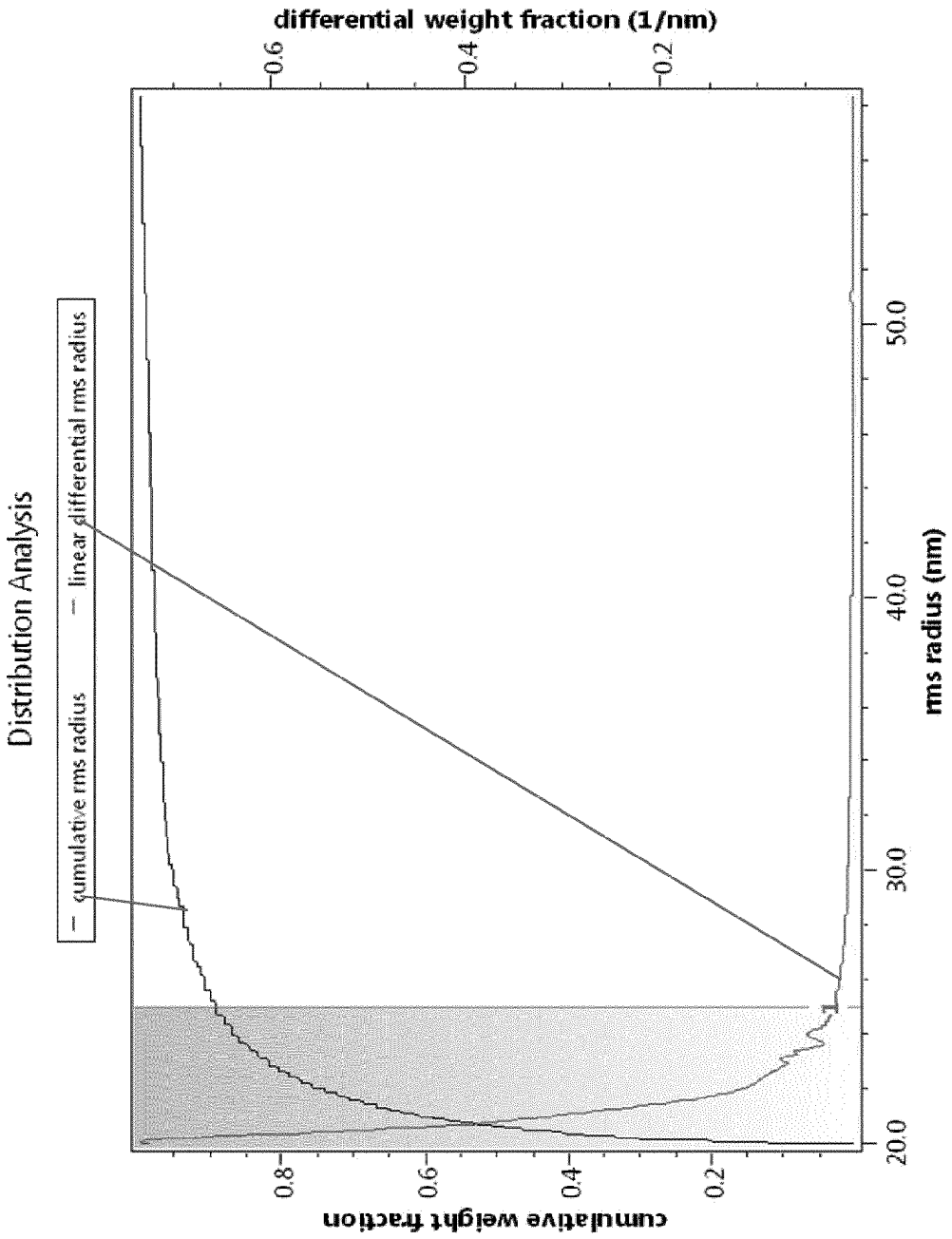
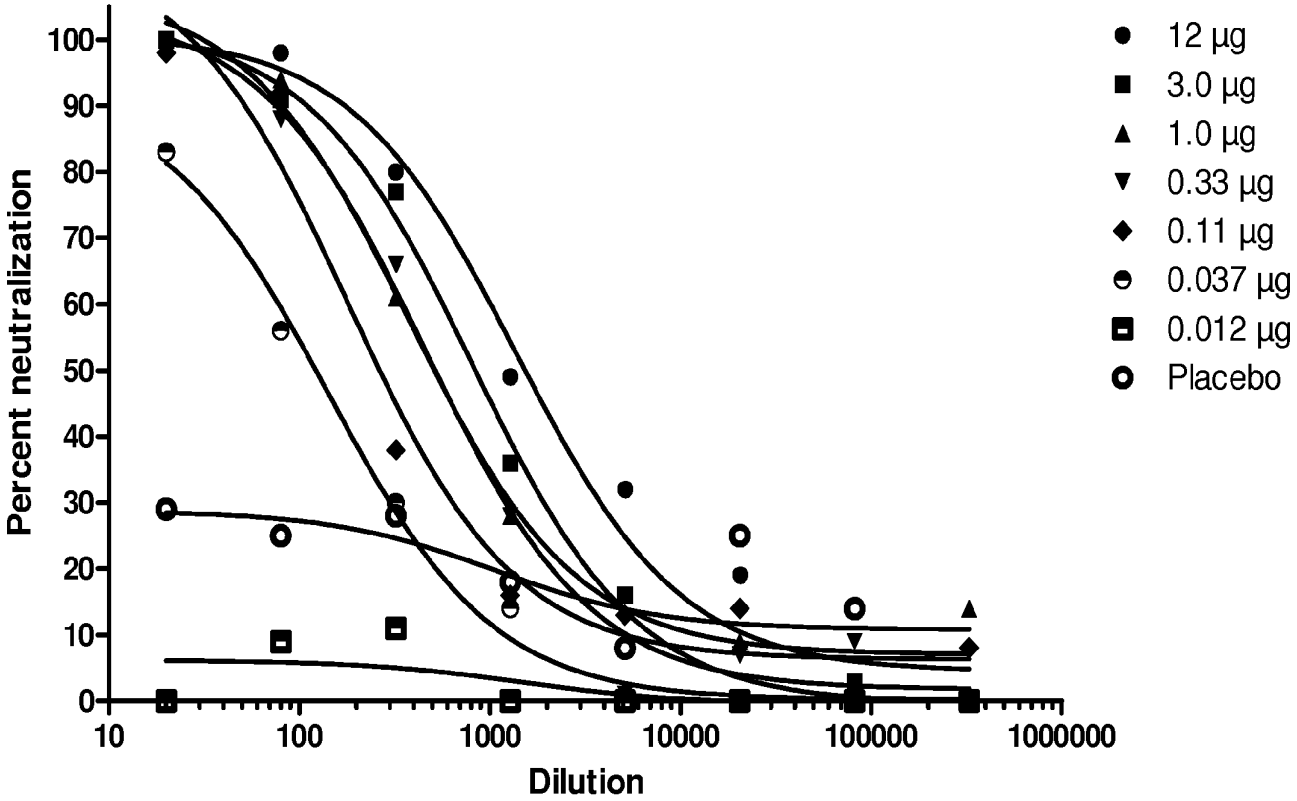
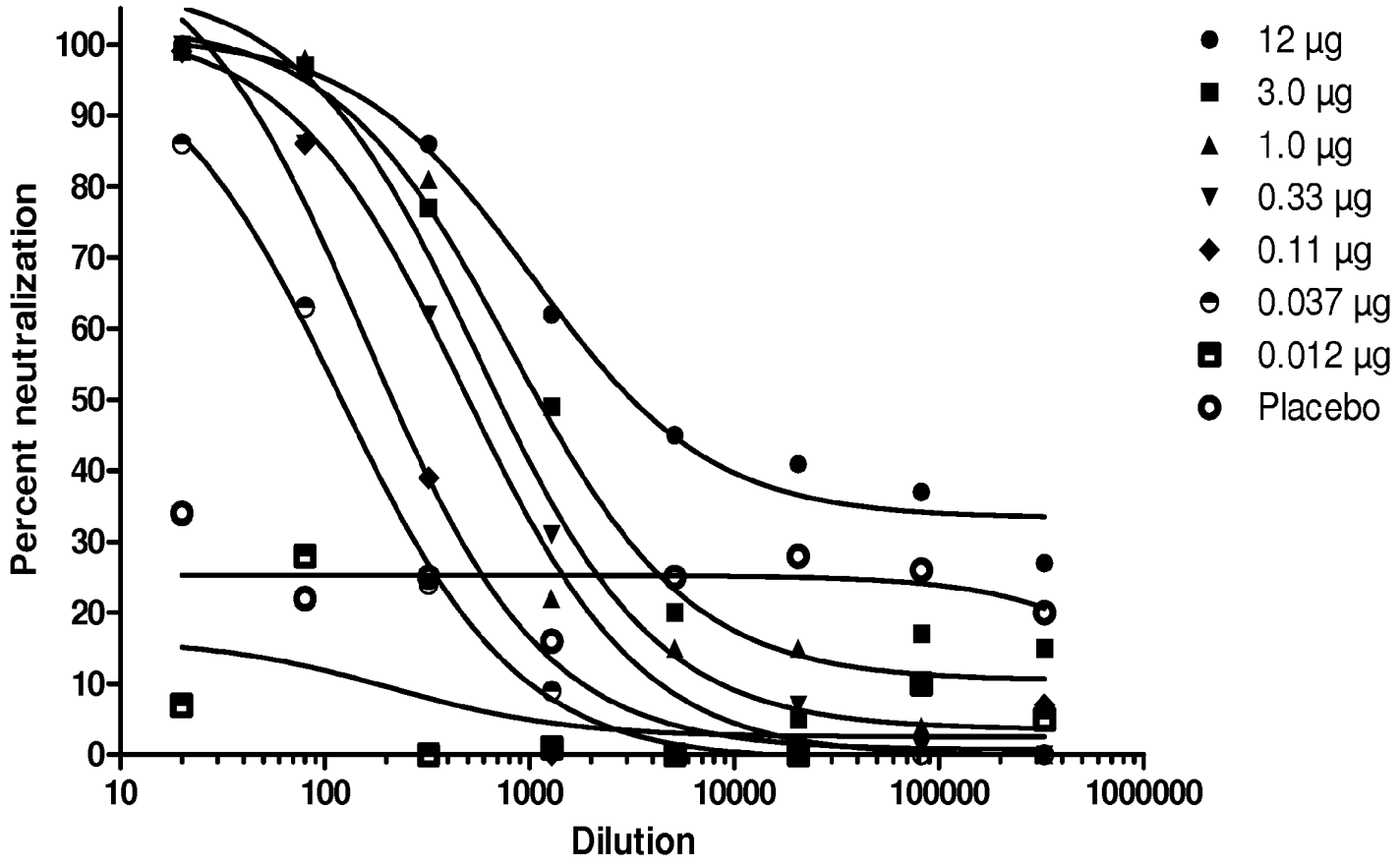

Distribution Analysis
cumulative rms radius
linear differential rms radius
differential weight fraction (1/nm)
0.6
0.4
0.2
cumulative weight fraction
0.8
0.6
0.4
0.2
20.0
30.0
40.0
50.0
rms radius (nm)

Figure 23



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1371	807.6	380.7	456.1	179.9	142.0

Figure 24



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1029	819.1	556.9	477.3	158.1	118.0

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DOCUMENT MADE AVAILABLE UNDER THE PATENT COOPERATION TREATY (PCT)

International application number:		**PCT/EP2016/082664**
International filing date:		**23 December 2016 (23.12.2016)**
Document type:		**Certified copy of priority document**
Document details:	Country/Office:	**EP**
	Number:	**16182845.4**
	Filing date:	**04 August 2016 (04.08.2016)**
Date of receipt at the International Bureau:		**16 February 2017 (16.02.2017)**

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-*bis*)

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PCT REQUEST

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0	**For receiving Office use only**	
0-1	International Application No.	**PCT/EP2016/082664**
0-2	International Filing Date	**23 DEC 2016 (23.12.2016)**
0-3	Name of receiving Office and "PCT International Application"	**RO/EP**

0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	PCT Online Filing Version 3.5.000.251e MT/FOP 20141031/0.20.5.20
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	European Patent Office (EPO) (RO/EP)
0-7	**Applicant's or agent's file reference**	P5706PC00
I	**Title of Invention**	Zika Virus Vaccine
II	**Applicant**	
II-1	This person is	Applicant only
II-2	Applicant for	All designated States
II-4	Name	Valneva SE
II-5	Address	World Trade Center Lyon Tour Oxygène 10-12 boulevard Marius Vivier Merle 69003 Lyon France
II-6	State of nationality	FR* *RO/EP
II-7	State of residence	FR
III-1	**Applicant and/or inventor**	
III-1-1	This person is	Inventor only
III-1-3	Inventor for	All designated States
III-1-4	Name (LAST, First)	BARBERO CALZADO, (Ms). Jana
III-1-5	Address	Valneva Austria GmbH Mittersteig 20/21 1050 Vienna Austria

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III-2	**Applicant and/or inventor**	
III-2-1	This person is	Inventor only
III-2-3	Inventor for	All designated States
III-2-4	Name (LAST, First)	NEBENFÜHR, (Mr). Mario
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III-3-1	This person is	Inventor only
III-3-3	Inventor for	All designated States
III-3-4	Name (LAST, First)	SCHLEGL, (Mr). Robert
III-3-5	Address	Valneva Austria GmbH Rosenthal 63 2500 Siegenfeld Austria
III-4	**Applicant and/or inventor**	
III-4-1	This person is	Inventor only
III-4-3	Inventor for	All designated States
III-4-4	Name (LAST, First)	WEBER, (Mr). Michael
III-4-5	Address	Valneva Austria GmbH Rechte Wienzeile 249/15 1120 Vienna Austria
III-5	**Applicant and/or inventor**	
III-5-1	This person is	Inventor only
III-5-3	Inventor for	All designated States
III-5-4	Name (LAST, First)	WRUSS, (Mr). Jürgen
III-5-5	Address	Valneva Austria GmbH Dampfgasse 25/27-29 1100 Vienna Austria

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IV-1	**Agent or common representative; or address for correspondence**	
	The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	Agent
IV-1-1	Name (LAST, First)	DEMPSTER, Robert
IV-1-2	Address	Maschio & Soames IP Limited 30 Carlton Crescent Southampton Hampshire SO15 2EW United Kingdom
IV-1-3	Telephone No.	02380 989270
IV-1-4	Facsimile No.	02381 781617
IV-1-5	e-mail	mail@maschiosoames.com
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	
VI-1	**Priority claim of earlier regional application**	
VI-1-1	Filing date	23 December 2015 (23.12.2015)
VI-1-2	Number	15202585.4
VI-1-3	Regional Office	EP
VI-2	**Priority claim of earlier regional application**	
VI-2-1	Filing date	18 March 2016 (18.03.2016)
VI-2-2	Number	16161068.8
VI-2-3	Regional Office	EP
VI-3	**Priority claim of earlier regional application**	
VI-3-1	Filing date	23 June 2016 (23.06.2016)
VI-3-2	Number	16176025.1
VI-3-3	Regional Office	EP
VI-4	**Priority claim of earlier regional application**	
VI-4-1	Filing date	23 June 2016 (23.06.2016)
VI-4-2	Number	16176049.1
VI-4-3	Regional Office	EP

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VI-5	**Priority claim of earlier regional application**		
VI-5-1	Filing date	04 August 2016 (04.08.2016)	
VI-5-2	Number	16182845.4	
VI-5-3	Regional Office	EP	
VI-6	**Priority document request**		
	The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1 VI-2 VI-3 VI-4 VI-5	
VI-7	**Incorporation by reference :**		
	where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.		
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	–	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	–	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	–	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	–	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	–	

PCT REQUEST

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IX	Check list	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description	101	✓
IX-3	Claims	2	✓
IX-4	Abstract	1	✓
IX-5	Drawings	21	✓
IX-6a	Sequence listing part of the description (also to be used for the purposes of international search)	–	✓
IX-7	TOTAL	130	
	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	–	✓
IX-20	**Figure of the drawings which should accompany the abstract**		
IX-21	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**	(PKCS7 Digital Signature)	
X-1-1	Name (LAST, First)	DEMPSTER, Robert	
X-1-3	Capacity (if such capacity is not obvious from reading the request)	(Representative)	

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	23 DEC 2016 (23.12.2016)
10-2	**Drawings:**	
10-2-1	Received	✓
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

CLAIMS

What is claimed is:

1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein said Zika virus vaccine is able to confer seroprotection on at least 70% of subjects that are administered the Zika virus vaccine.

2. The Zika virus vaccine of claim 1, wherein the Zika virus particle is able to confer seroprotection on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects that are administered the Zika virus vaccine, preferably on at least 80% of subjects.

3. The vaccine of claim 1 or 2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

4. The vaccine of any one of claims 1 to 3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

5. The vaccine of any one of claims 1 to 4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

6. The vaccine of claim 5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

7. The vaccine of claim 6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

8. The vaccine of claim 7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

9. The vaccine of any one of claims 5 to 8, wherein the chemical activation is performed at about +4°C or about +22°C.

10. The vaccine of any one of claims 1 to 9, further comprising an adjuvant.

11. The vaccine of claim 10, wherein the adjuvant is an aluminum salt adjuvant.

12. The vaccine of claim 11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

13. The vaccine of any one of claims 10 to 12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

14. The vaccine of claim 13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

15. The vaccine of any one of claims 1 to 14, further comprising one or more pharmaceutically acceptable excipients.

16. The vaccine of any one of claims 1 to 15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 µg/mL, especially below 100 ng/mL.

17. The vaccine of claim 16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

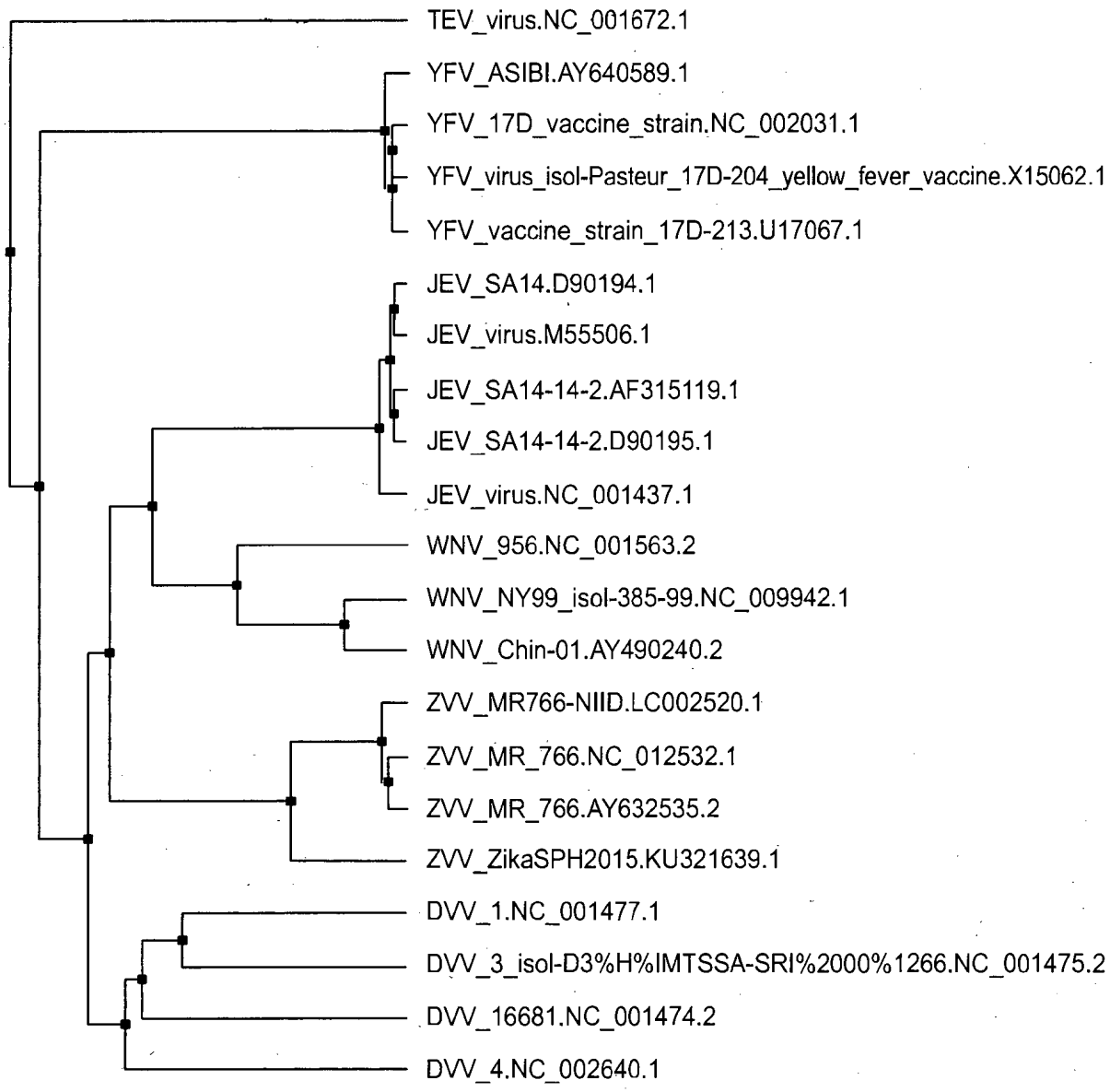

TEV_virus.NC_001672.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
DVV_1.NC_001477.1
DVV_3_isol-D3%H%IMTSSA-SRI%2000%1266.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1

Figure 1

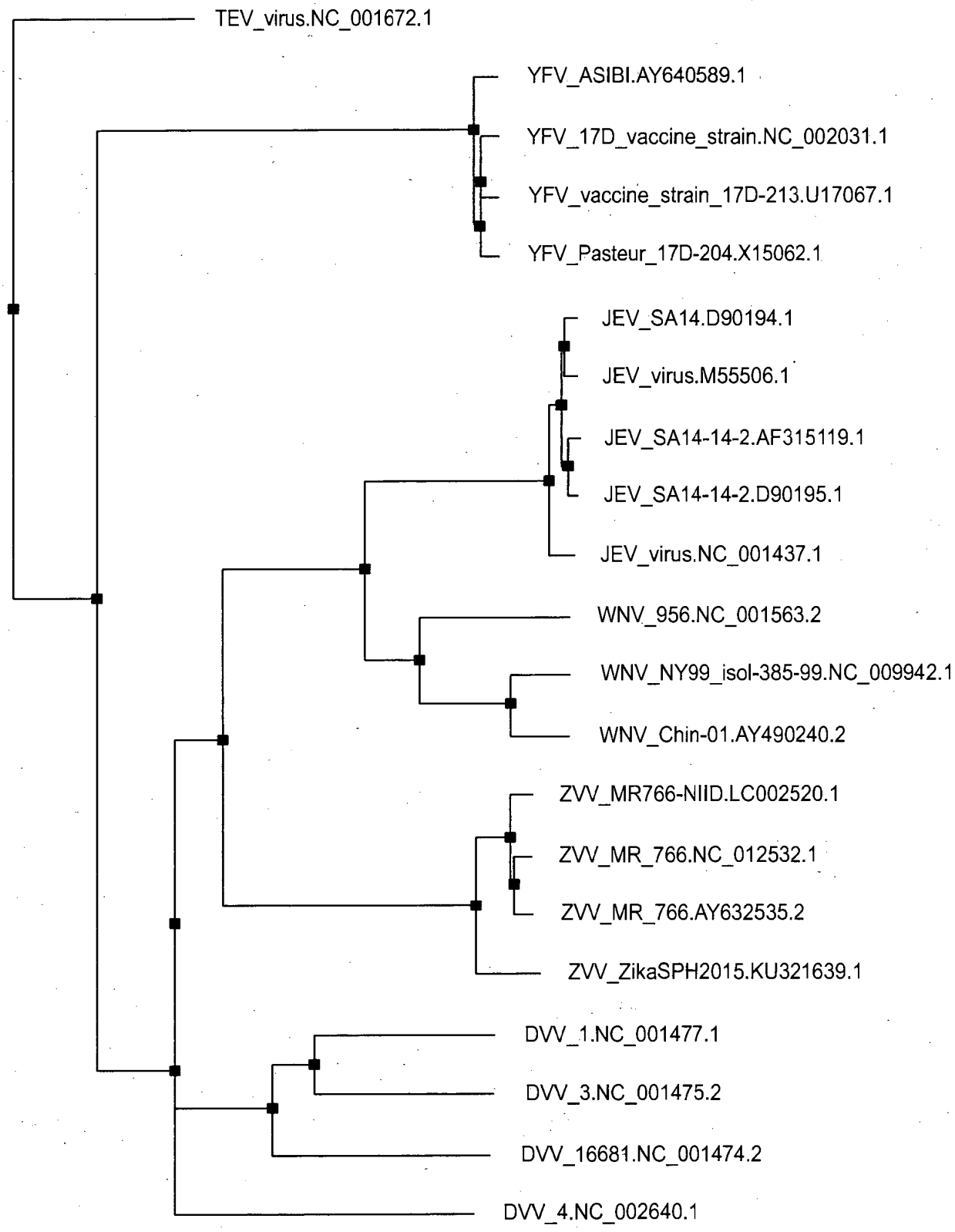

TEV_virus.NC_001672.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_vaccine_strain_17D-213.U17067.1
YFV_Pasteur_17D-204.X15062.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
DVV_1.NC_001477.1
DVV_3.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1

Figure 2

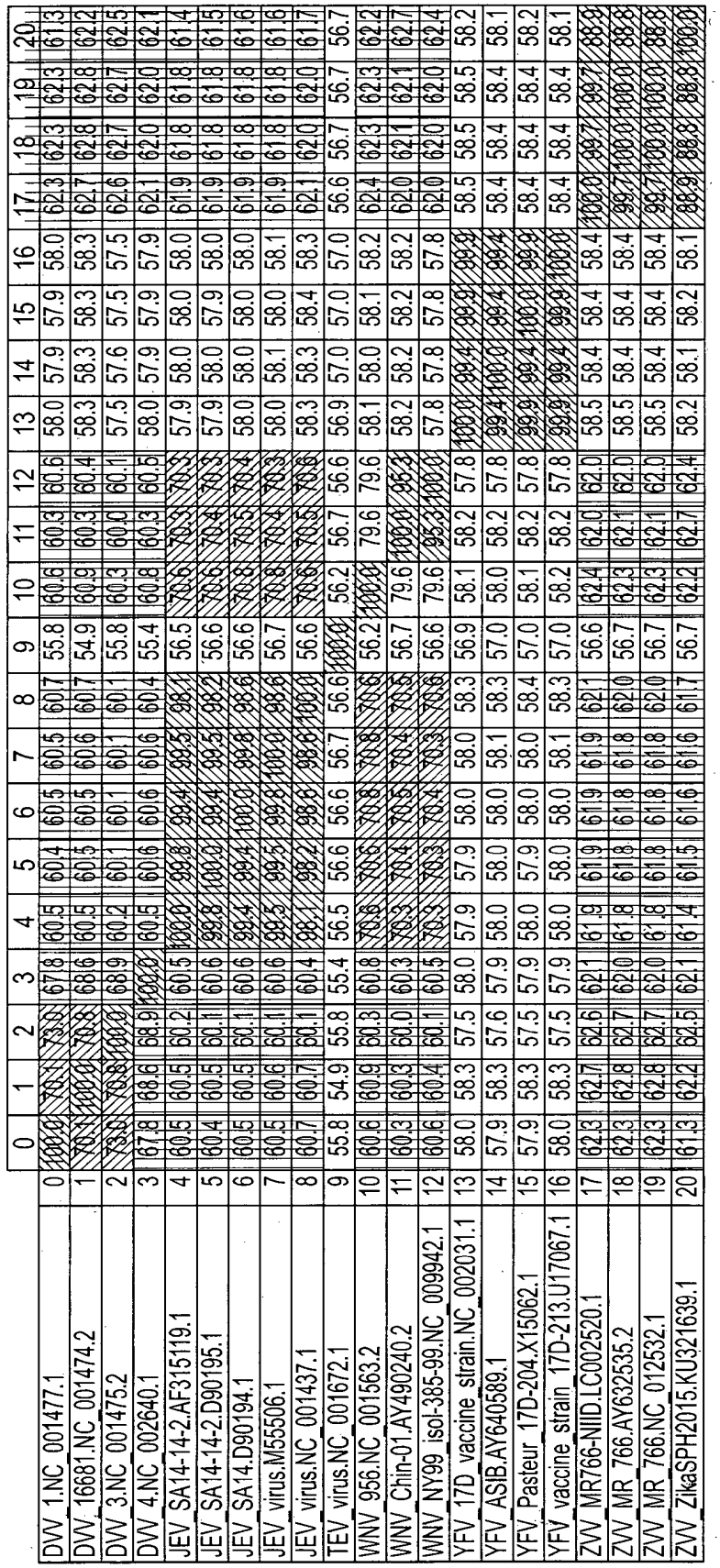

% Identity and higher
% Identity and higher
% Identity and higher

Figure 3

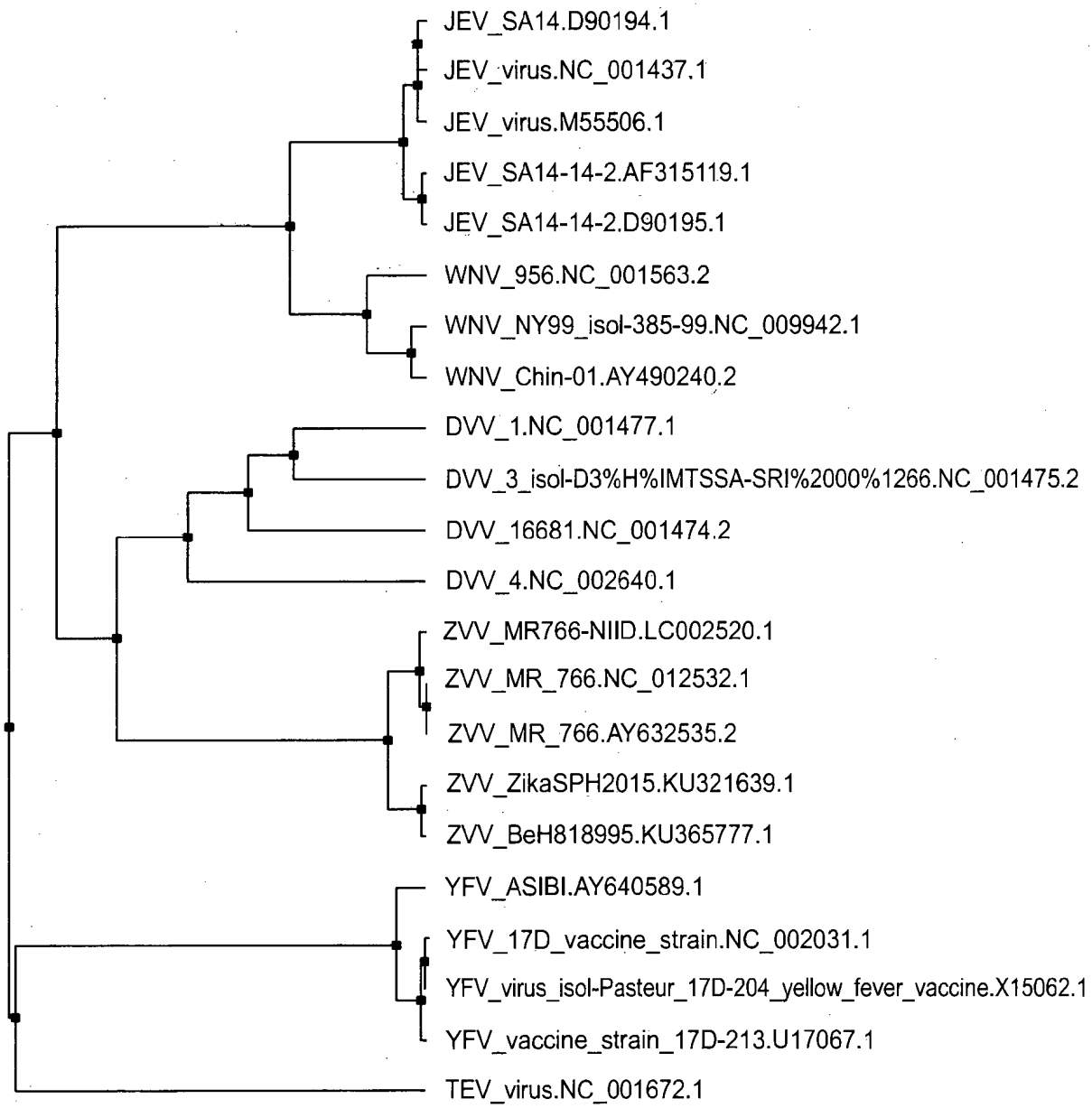

JEV_SA14.D90194.1
JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
DVV_1.NC_001477.1
DVV_3_isol-D3%H%IMTSSA-SRI%2000%1266.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
ZVV_BeH818995.KU365777.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1

Figure 4

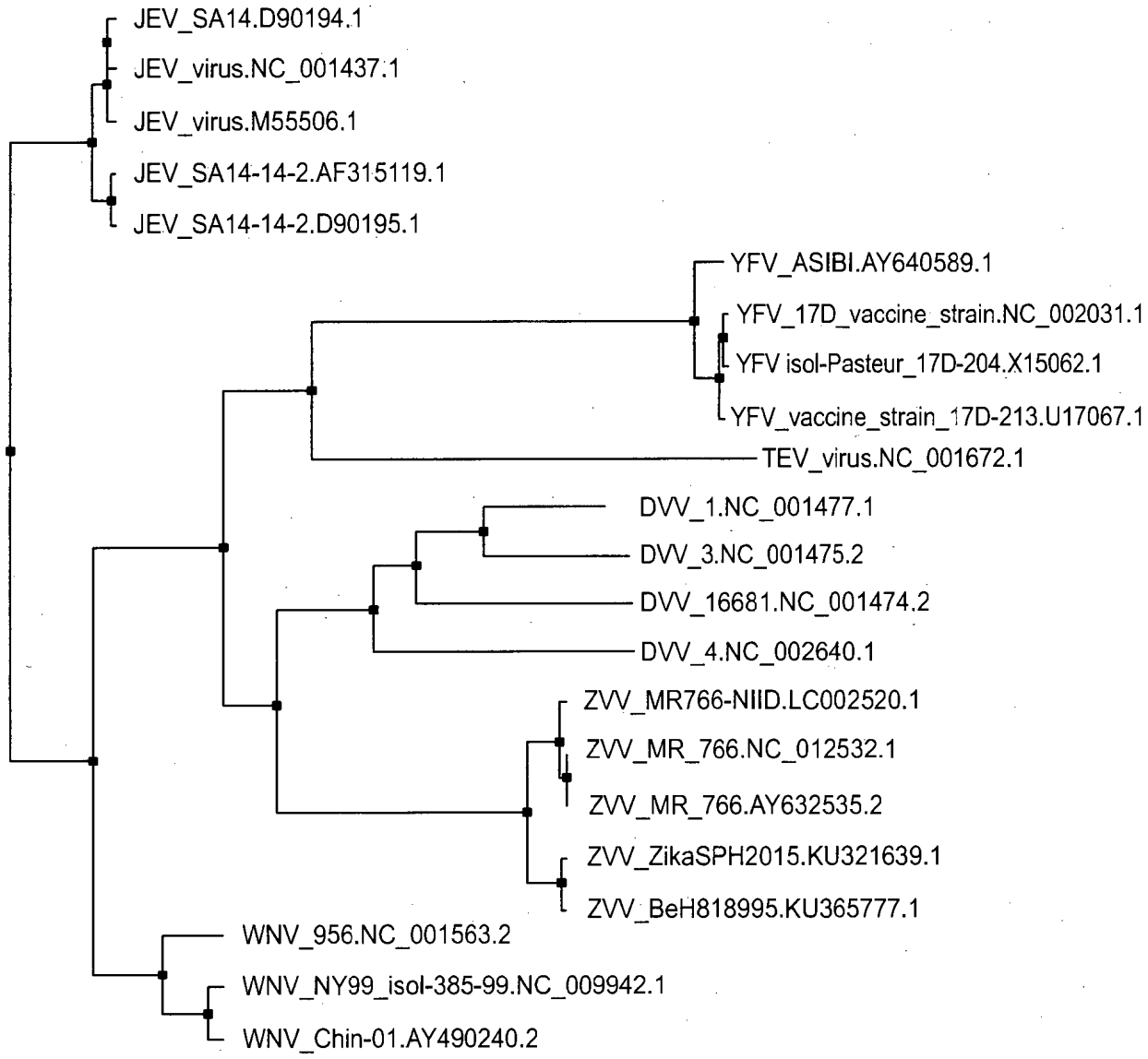

JEV_SA14.D90194.1
JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV isol-Pasteur_17D-204.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1
DVV_1.NC_001477.1
DVV_3.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
ZVV_BeH818995.KU365777.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2

Figure 5

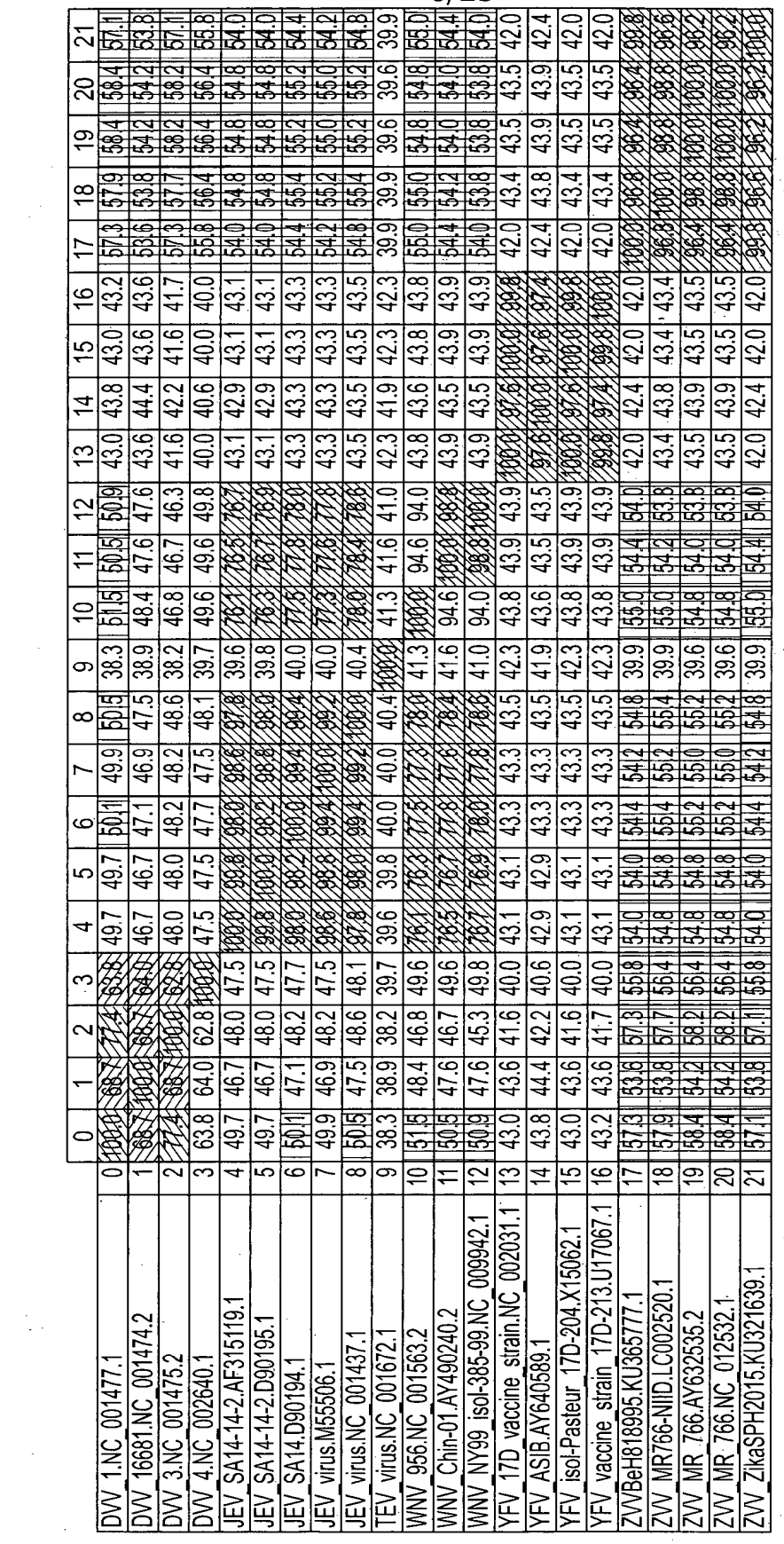

50 % Identity and higher
60 % Identity and higher
70 % Identity and higher

Figure 6

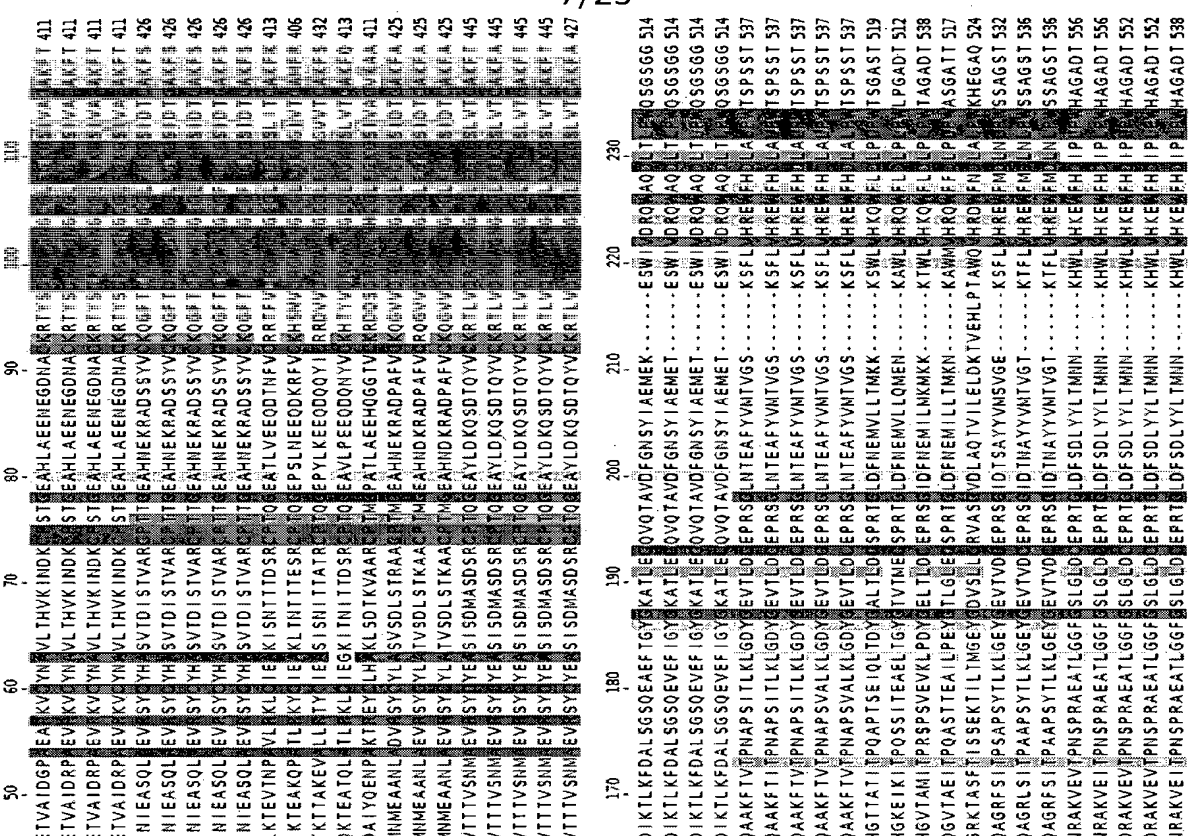


Figure 7 (Part 1 of 3)

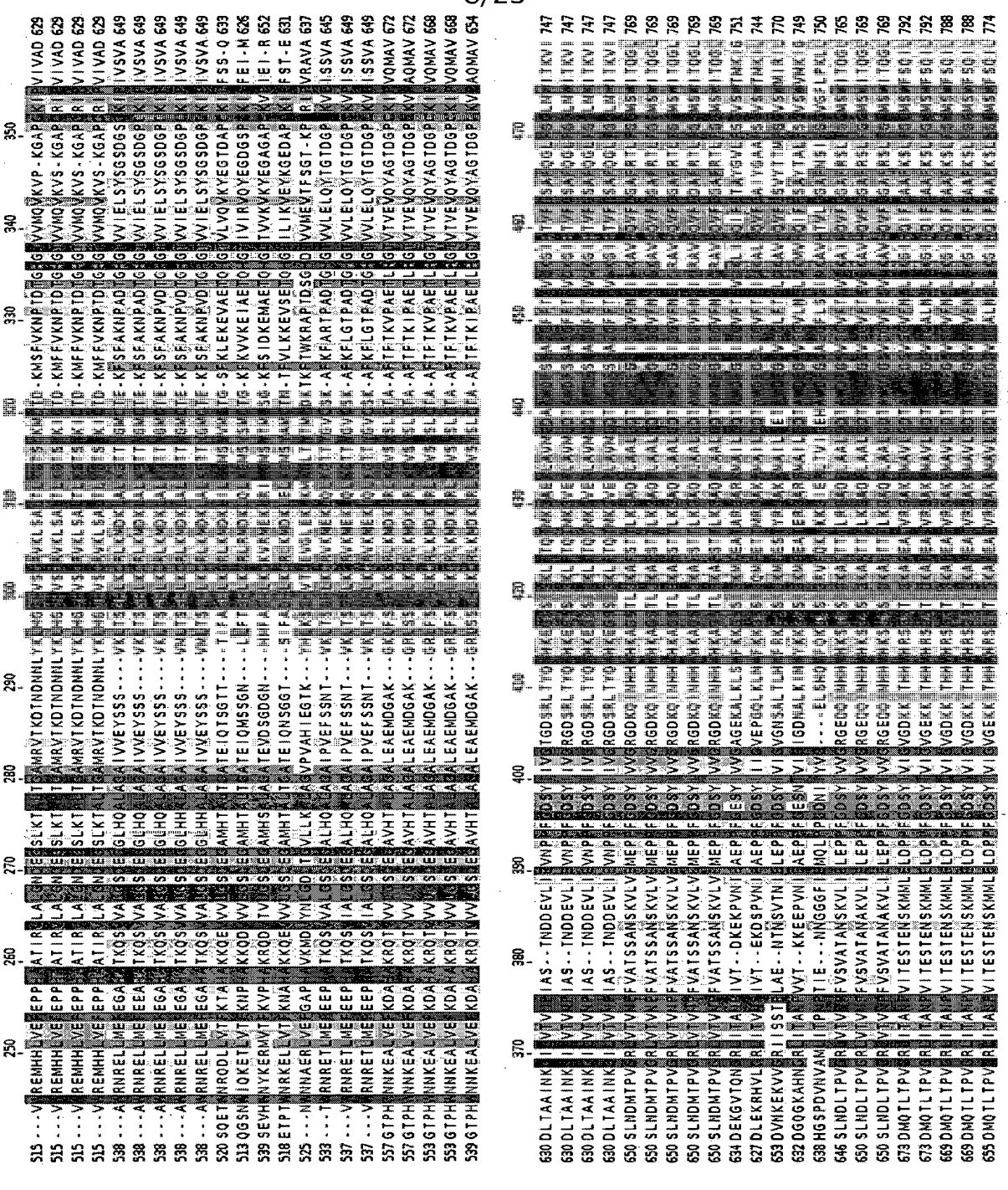


Figure 7 (Part 2 of 3)

Figure 7 (Part 3 of 3)

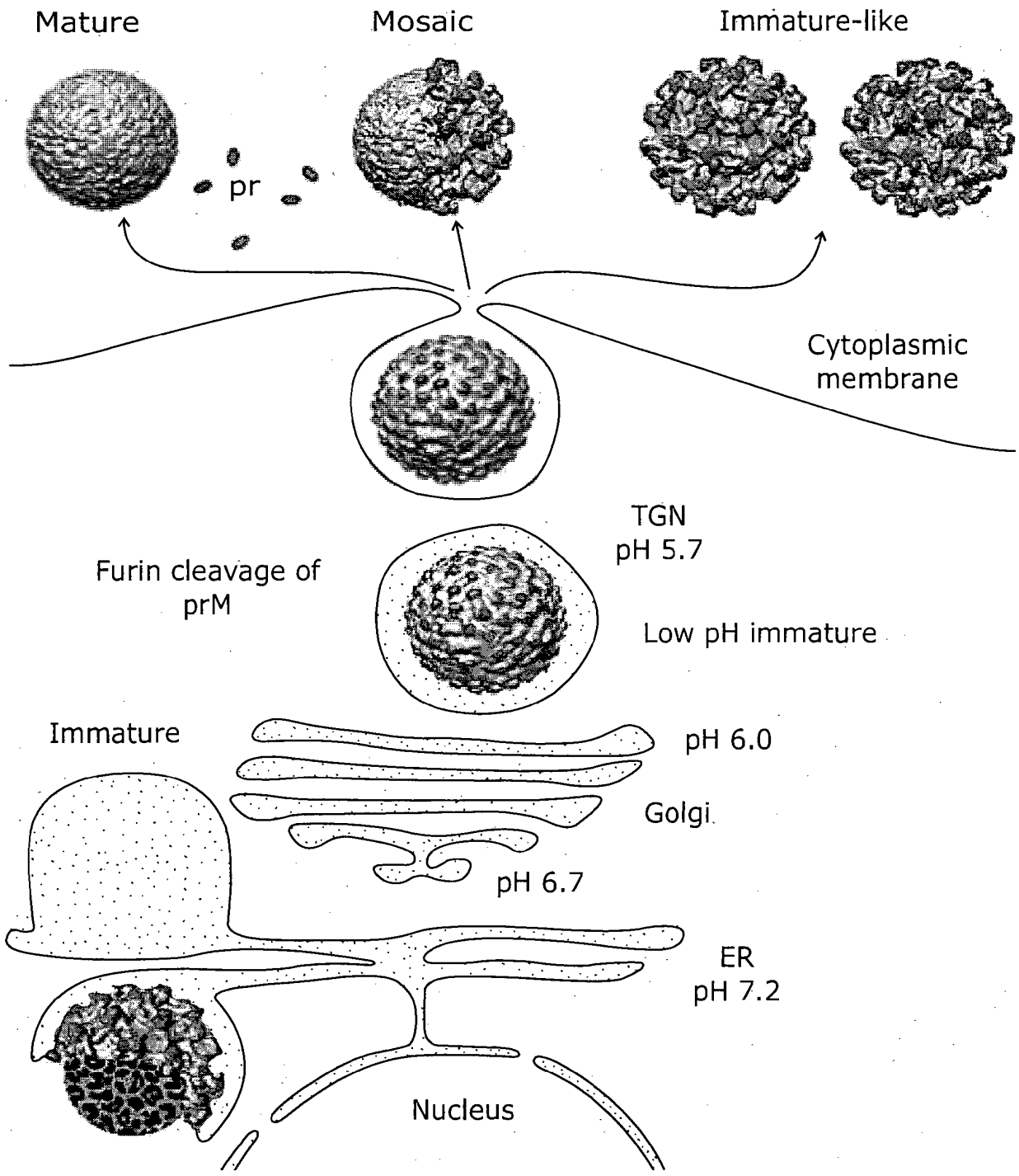

Mature
Mosaic
Immature-like
pr
Cytoplasmic membrane
TGN
pH 5.7
Furin cleavage of prM
Low pH immature
Immature
pH 6.0
Golgi
pH 6.7
ER
pH 7.2
Nucleus

Figure 8

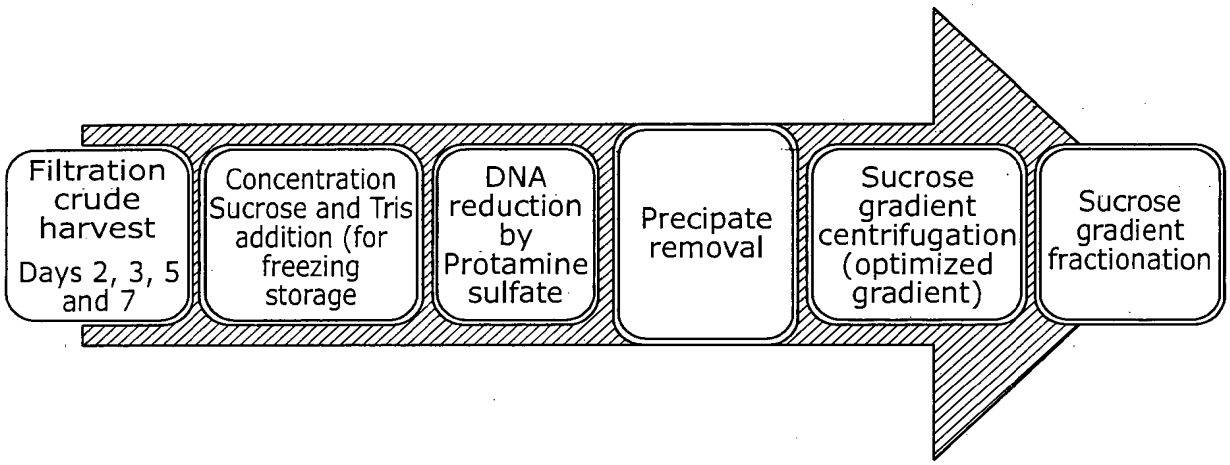


Figure 9A

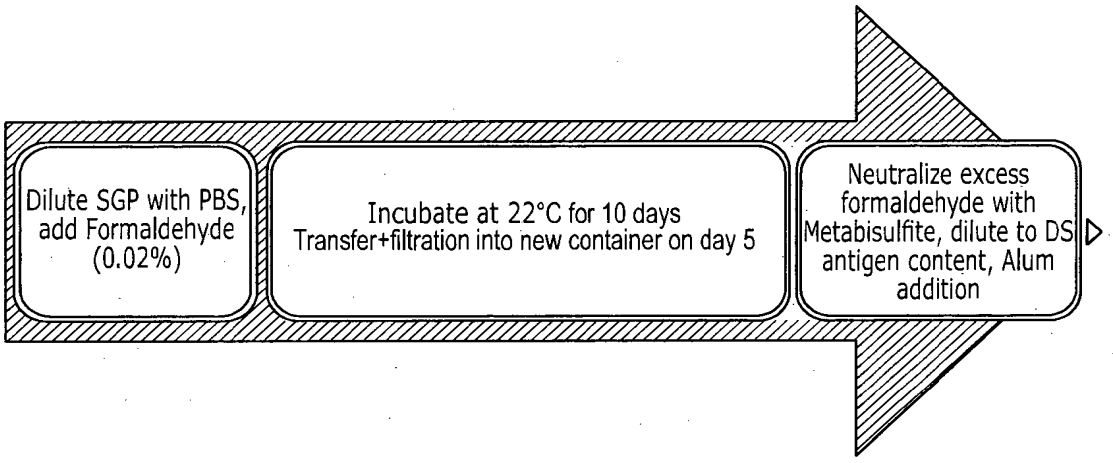


Figure 9B

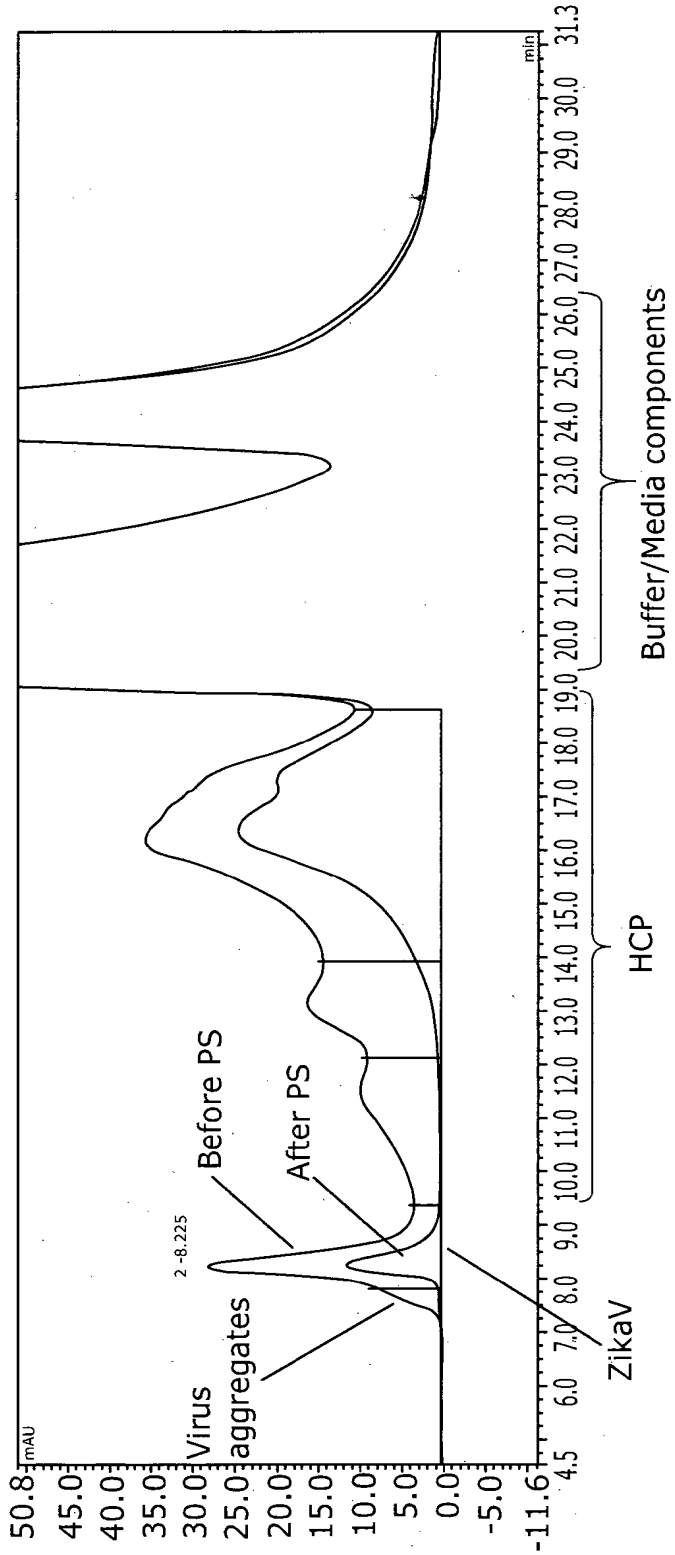
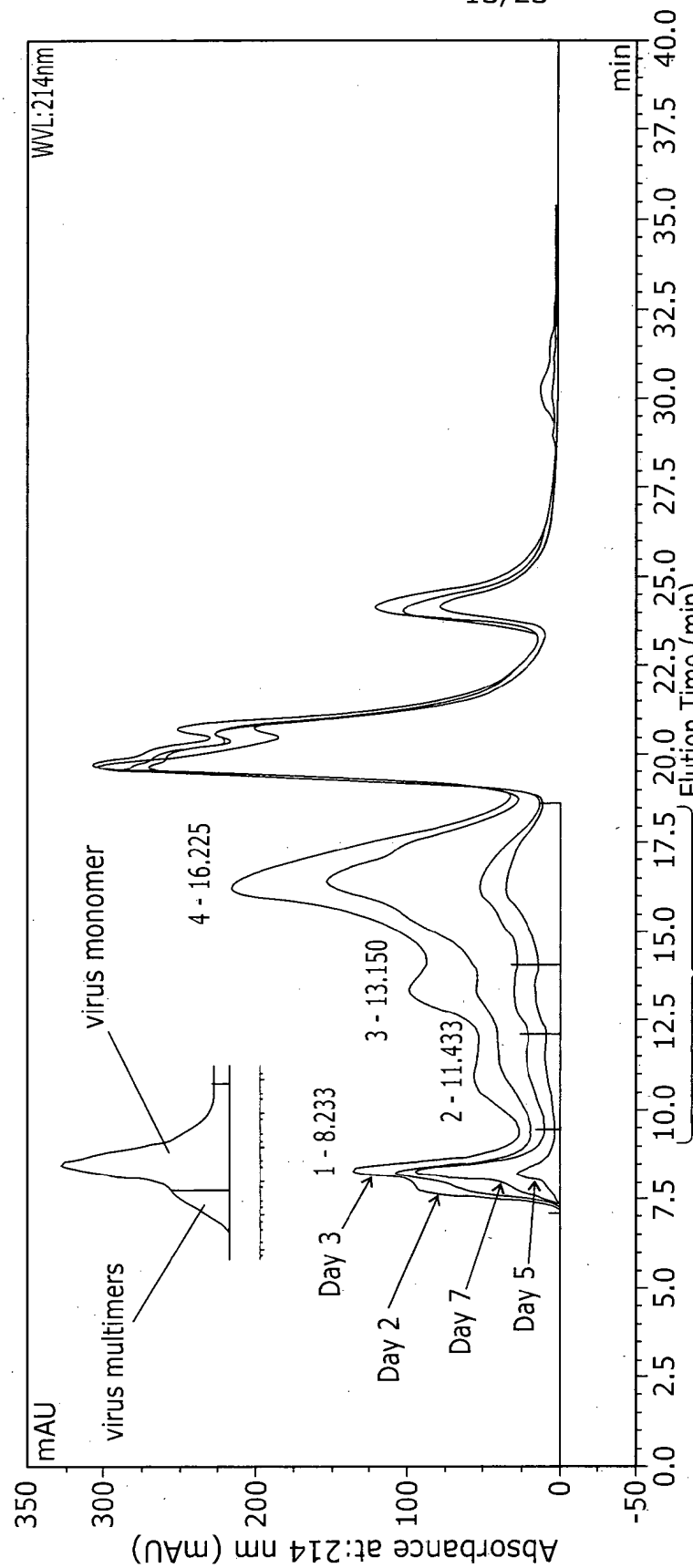

mAU
50.8
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
-11.6
min
4.5
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0
21.0
22.0
23.0
24.0
25.0
26.0
27.0
28.0
29.0
30.0
31.3
2 -8.225
Virus aggregates
Before PS
After PS
ZikaV
HCP
Buffer/Media components

Figure 10



+ Similar virus concentration in harvests Day 2, Day3, Day 7; lower virus concentration in harvest Day 5
+ Day 2 and Day 3 harvests contained more HCP than Days 5/7 (correlates with observed CPE)
+ Minor amount of virus multimers observed

Figure 11

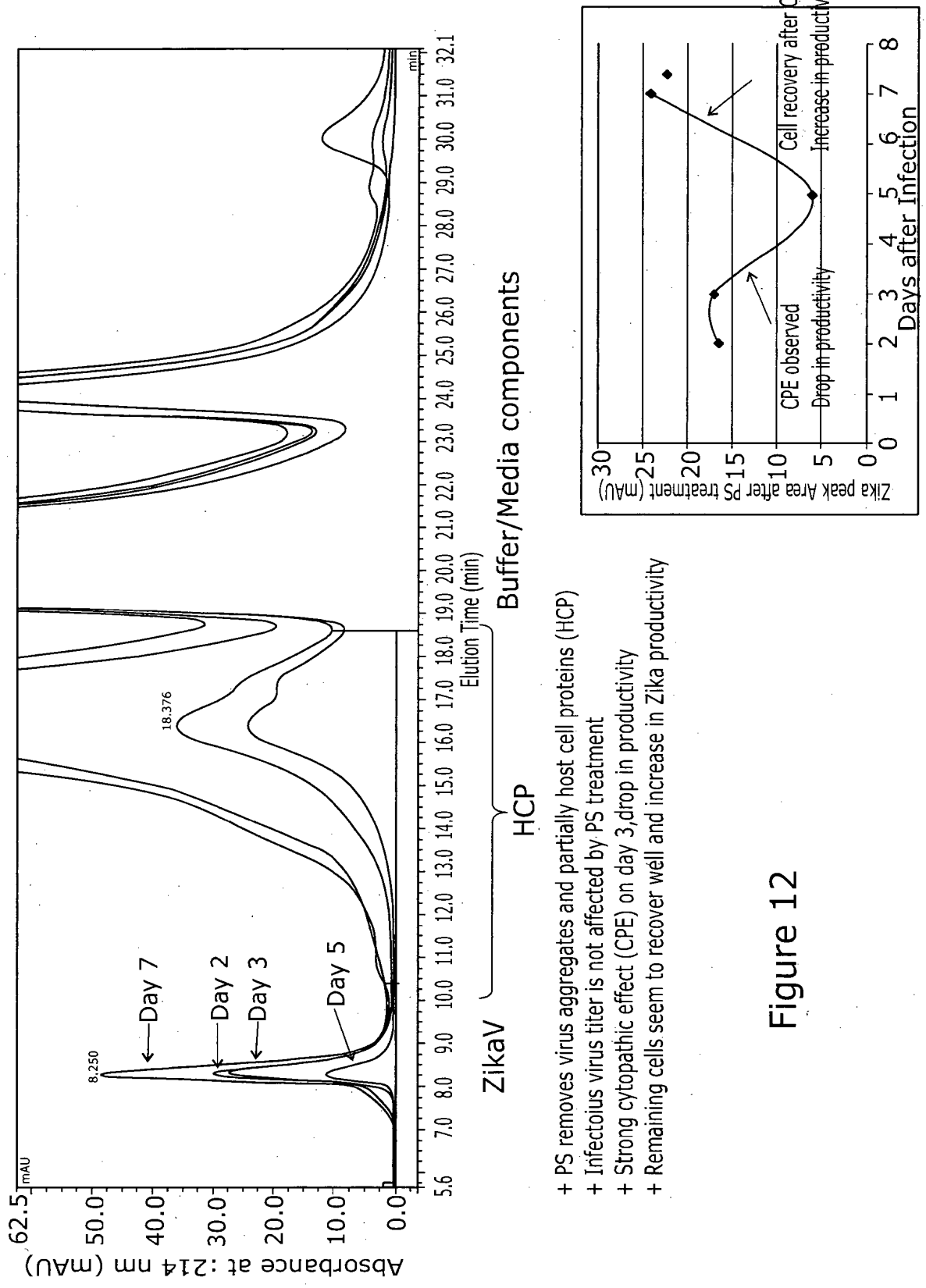

Absorbance at :214 nm (mAU)
mAU
62.5
50.0
40.0
30.0
20.0
10.0
0.0
8.250
18.376
Day 7
Day 2
Day 3
Day 5
5.6 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0 31.0 32.1
min
Elution Time (min)
ZikaV
HCP
Buffer/Media components
+ PS removes virus aggregates and partially host cell proteins (HCP)
+ Infectoius virus titer is not affected by PS treatment
+ Strong cytopathic effect (CPE) on day 3,drop in productivity
+ Remaining cells seem to recover well and increase in Zika productivity
Zika peak Area after PS treatment (mAU)
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8
Days after Infection
CPE observed
Drop in productivity
Cell recovery after CPE
Increase in productivity

Figure 12

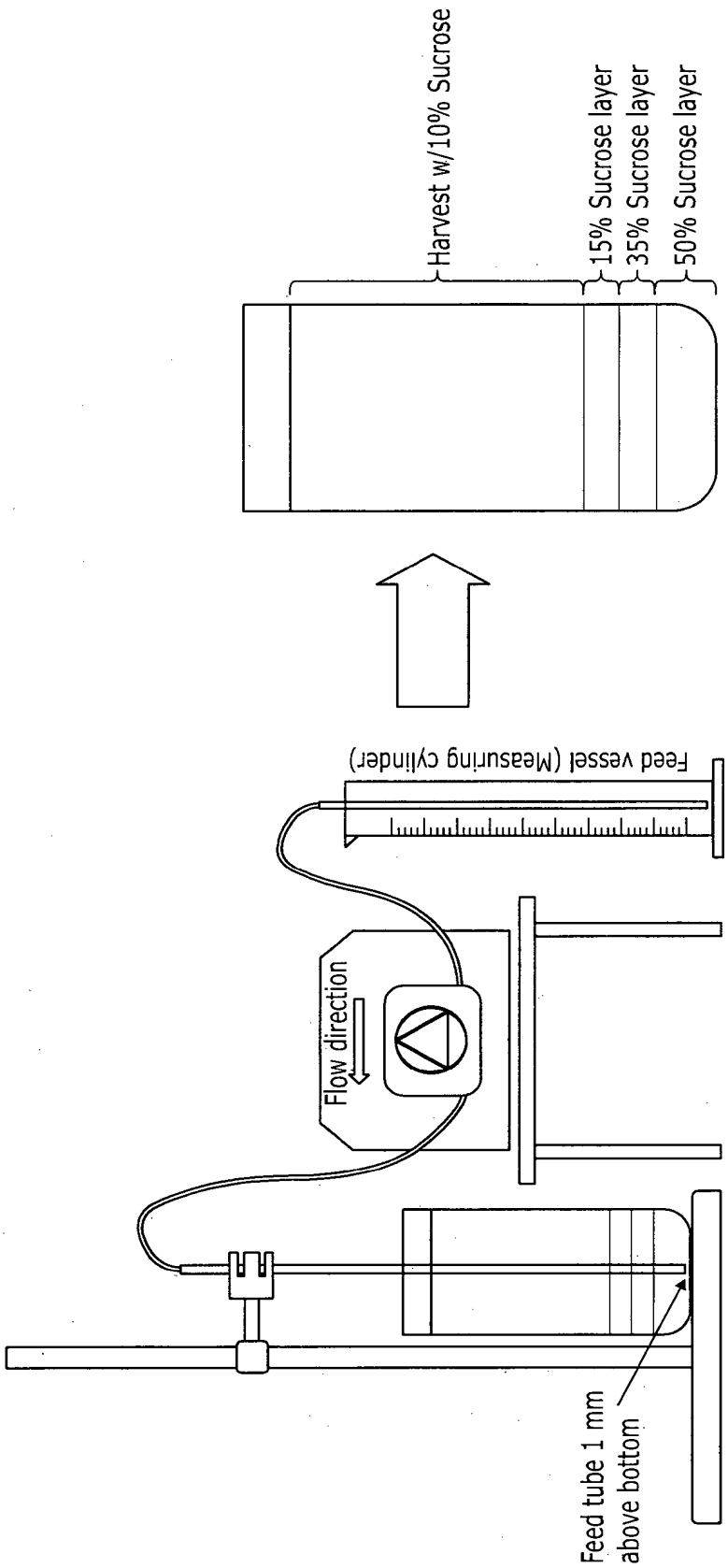
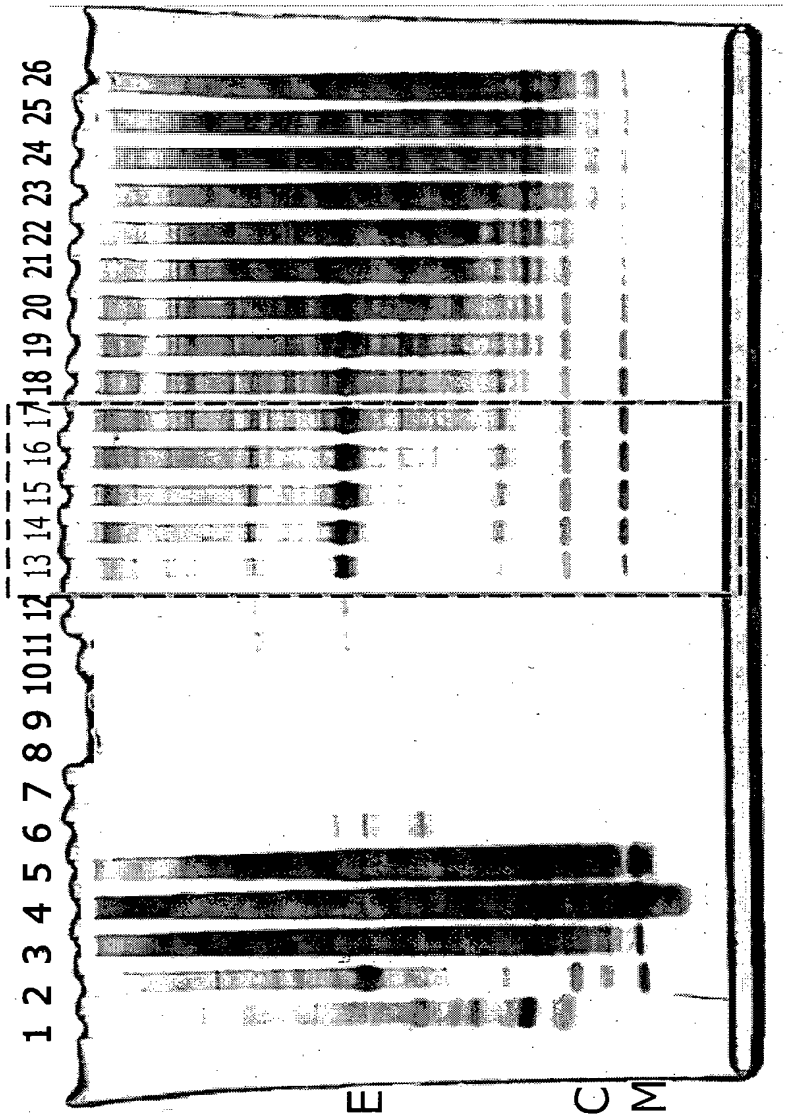

Harvest w/10% Sucrose
15% Sucrose layer
35% Sucrose layer
50% Sucrose layer
Feed vessel (Measuring cylinder)
Flow direction
Feed tube 1 mm above bottom

Figure 13

Lane	Sample
1	Molecular weight Marker
2	JEV Sucrose Gradient Pool (Reference)
3	Zika Harvest pool (day 2,3,5,7)
4	Zika Harvest pool (day 2,3,5,7)concentrated 300x
5	Zika Harvest pool (day 2,3,5,7)concentrated 300x + Protamine sulfate
6	Ultrafiltration Permeate
7	SGC Bottle 1, Fraction 4
8	SGC Bottle 1, Fraction 5
9	SGC Bottle 1, Fraction 6
10	SGC Bottle 1, Fraction 7
11	SGC Bottle 1, Fraction 8
12	SGC Bottle 1, Fraction 9
13	SGC Bottle 1, Fraction 10
14	SGC Bottle 1, Fraction 11
15	SGC Bottle 1, Fraction 12
16	SGC Bottle 1, Fraction 13
17	SGC Bottle 1, Fraction 14
18	SGC Bottle 1, Fraction 15
19	SGC Bottle 1, Fraction 16
20	SGC Bottle 1, Fraction 17
21	SGC Bottle 1, Fraction 18
22	SGC Bottle 1, Fraction 19
23	SGC Bottle 1, Fraction 20
24	SGC Bottle 1, Fraction 21
25	SGC Bottle 1, Fraction 22
26	SGC Bottle 1, Fraction 23



Pooled fractions F10-F14
(10mL total volume pere bottle)

Figure 14

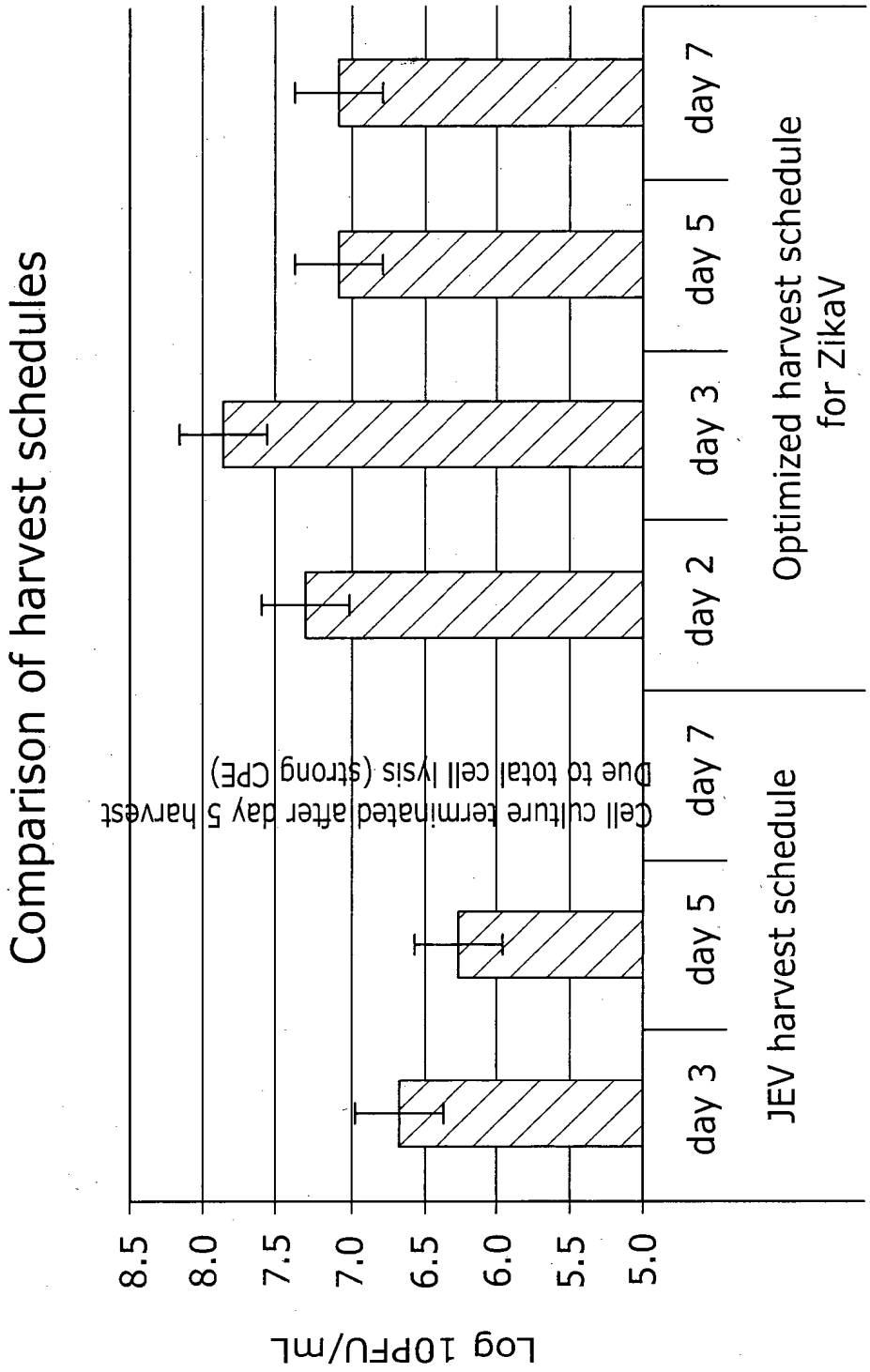

Comparison of harvest schedules
Log 10PFU/mL
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
day 3
day 5
day 7
JEV harvest schedule
Cell culture terminated after day 5 harvest
Due to total cell lysis (strong CPE)
day 2
day 3
day 5
day 7
Optimized harvest schedule
for ZikaV

Figure 15

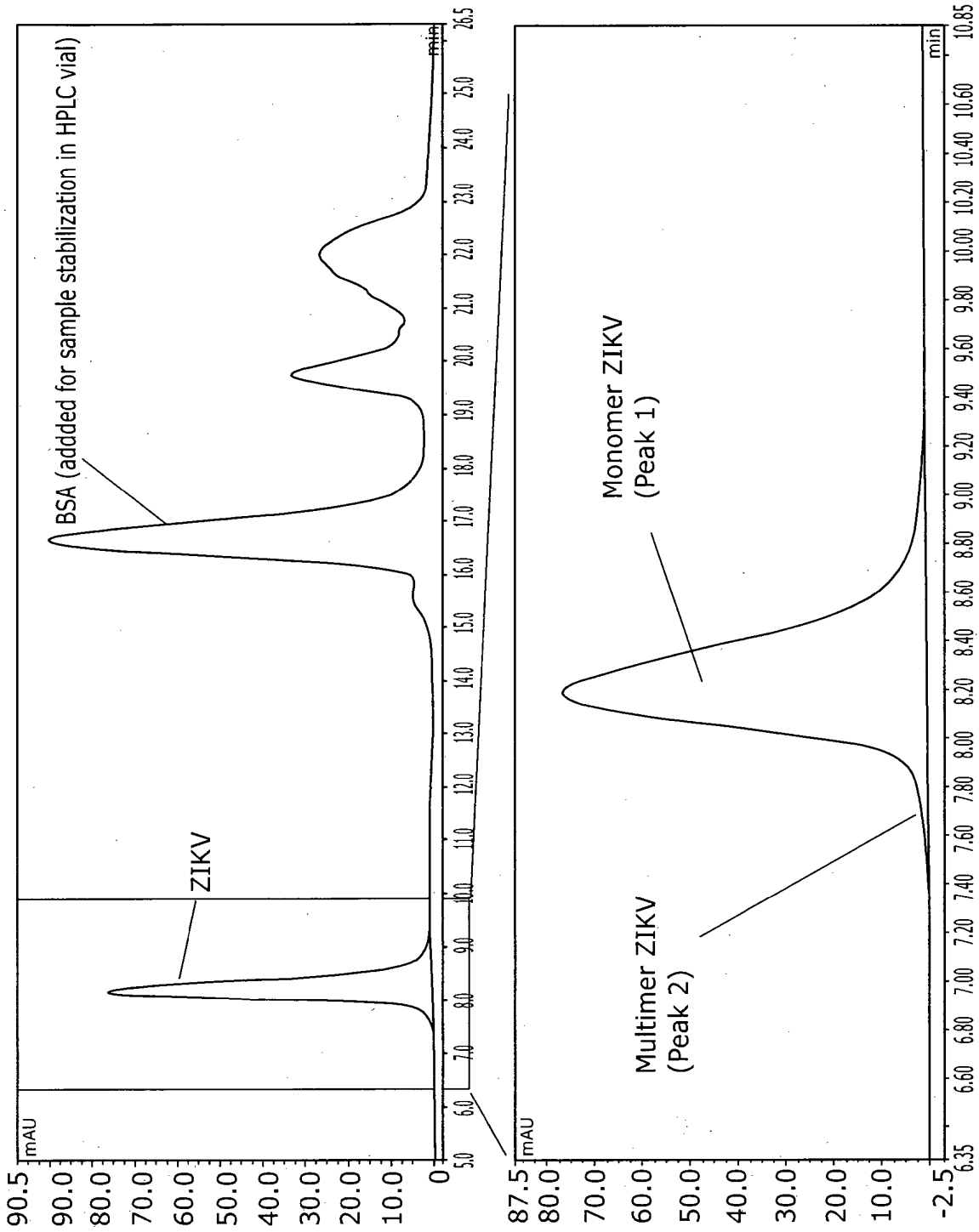

BSA (addded for sample stabilization in HPLC vial)
ZIKV
mAU
min
Monomer ZIKV
(Peak 1)
Multimer ZIKV
(Peak 2)
mAU
min

Figure 16

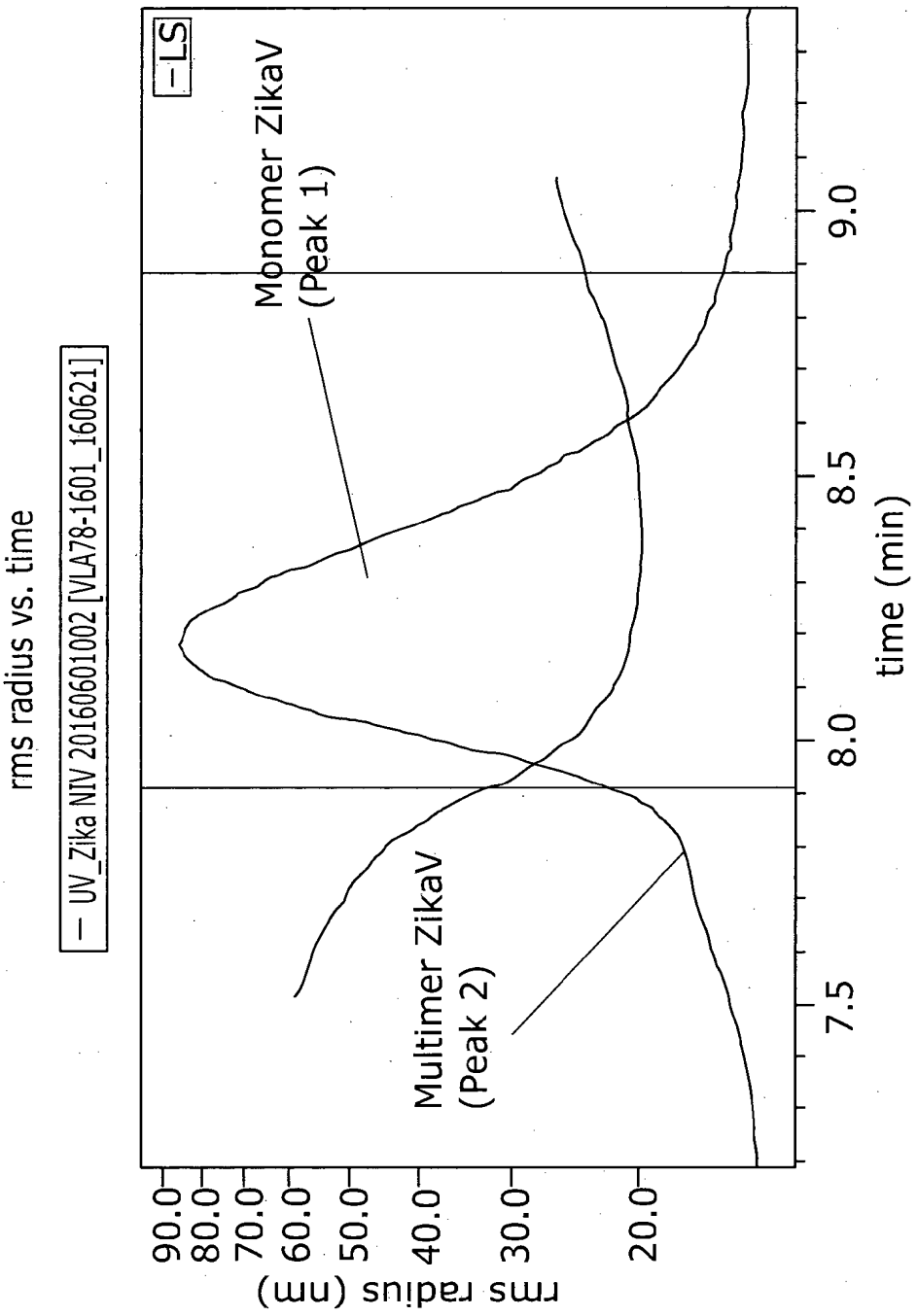

rms radius vs. time
UV_Zika NIV 20160601002 [VLA78-1601_160621]
LS
Monomer ZikaV
(Peak 1)
Multimer ZikaV
(Peak 2)
rms radius (nm)
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
7.5
8.0
8.5
9.0
time (min)

Figure 17

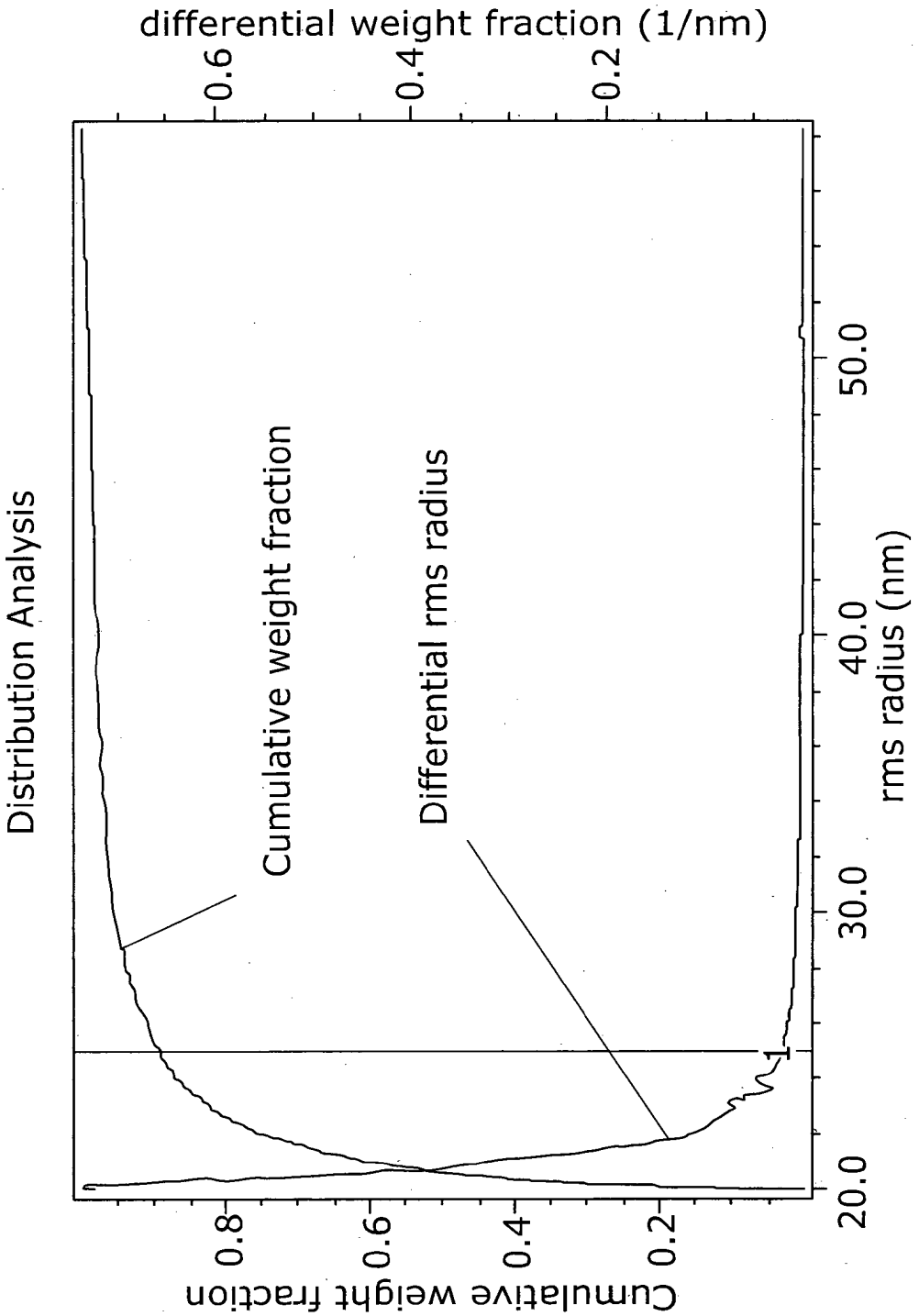
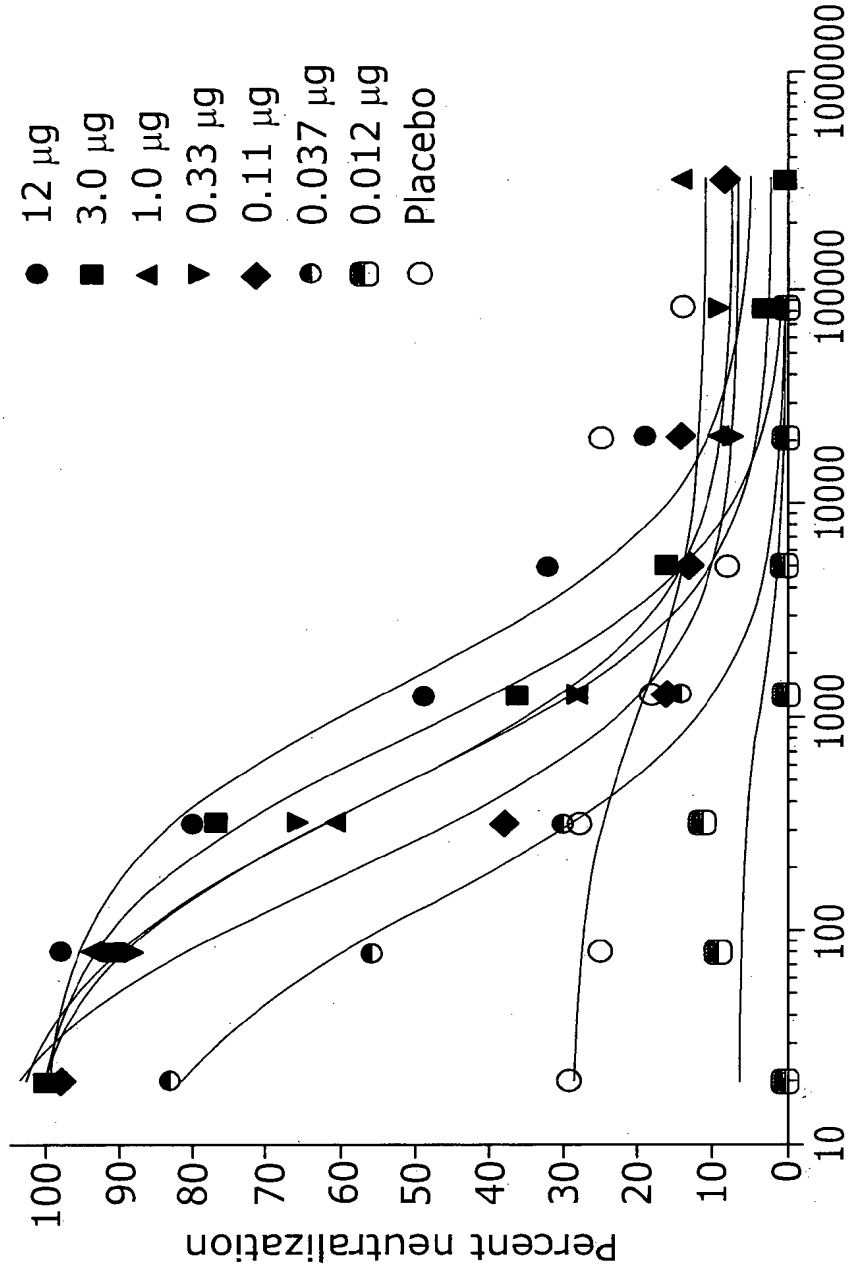
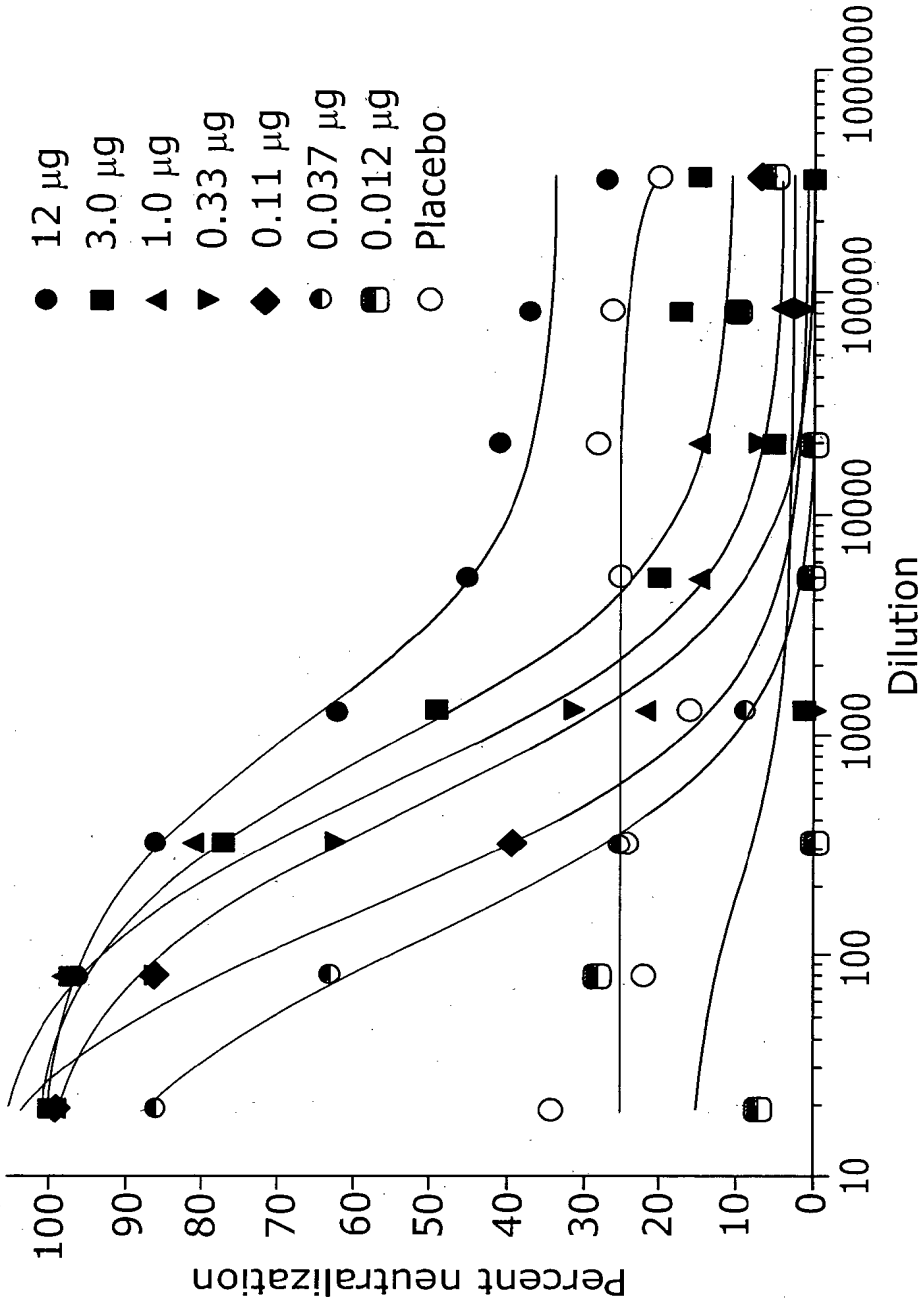

Distribution Analysis
Cumulative weight fraction
Differential rms radius
Cumulative weight fraction
0.8
0.6
0.4
0.2
20.0
30.0
40.0
50.0
rms radius (nm)
differential weight fraction (1/nm)
0.6
0.4
0.2

Figure 18



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1371	807.6	380.7	456.1	179.9	142.0

Figure 19



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1029	819.1	556.9	477.3	158.1	118.0

Figure 20

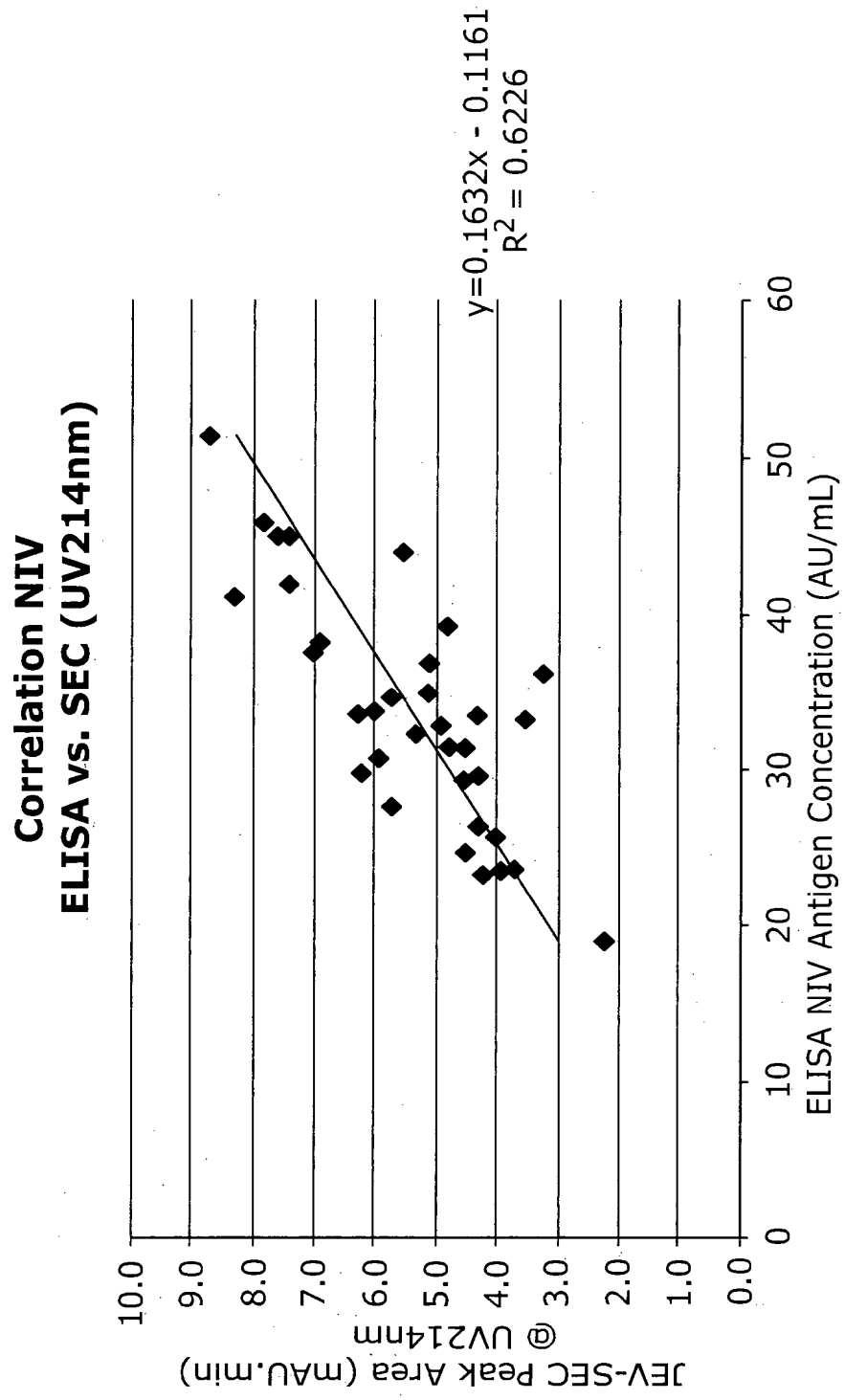

Correlation NIV
ELISA vs. SEC (UV214nm)
y=0.1632x - 0.1161
R2 = 0.6226
JEV-SEC Peak Area (mAU.min) @ UV214nm
ELISA NIV Antigen Concentration (AU/mL)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
10
20
30
40
50
60

Figure 21

ZIKA VIRUS VACCINE

FIELD OF INVENTION

The disclosure relates to methods for the purification of Zika viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate. The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site http://www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including

Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measures involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

A preventative vaccine against Zika virus is a pressing medical need in endemic areas and in geographical areas where the vector is spreading. Furthermore, as Zika infection has dire consequences on embryonic and fetal development, a safe and effective vaccine for women of child-bearing potential or pregnant women is needed. Vaccines administered during pregnancy must be very safe for both the mother and the developing fetus. While live attenuated viral vaccines are highly effective, they are often not considered safe enough for administration to pregnant women. In this regard, inactivated viral vaccines, which lack the ability to propagate in the vaccinated subject, are considered much safer. Development of an inactivated Zika virus vaccine for administration to at-risk patients would fill this need.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can be very efficiently separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to Zika virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely

devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Detail experimental examples to the above are provided for Zika virus.

The herein disclosed in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was unexpectedly higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, Nature doi:10.1038/nature18952.). Inactivated viruses are among the safest vaccines and especially preferred for delivery to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figure 7: Alignment (shading: % identity, aa), E-protein.

Figure 8: An example of virus particle maturation in the host cell. As observed in flaviviruses, full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID50 activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 9: An exemplary downstream virus purification process from the crude harvest to formulation of the drug substance (vaccine), a preferred embodiment of the process of the invention (**A**). A flow-chart of an exemplary virus inactivation process is shown in (**B**).

Figure 10: PS treatment results in selective removal of Zika virus aggregates and Vero HCP and LMW impurities (SEC-HPLC of 30x concentrated Zika Virus harvest day 5).

Figure 11: SEC-HPLC of individual 30x concentrated Zika harvest prior to PS treatment at different time points.

Figure 12: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment at different time points. The smaller graph indicates the observed cytopathic effect (CPE) over time.

Figure 13: Preparation of the sucrose gradient.

Figure 14: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 15: Comparison of JEV and ZikaV harvest schedules/yields.

Figure 16: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column. Both panels are from the same chromatogram. The upper graph is the complete elution profile; the lower graph is an enlargement of the ZIKAV elution peak.

Figure 17: SEC-MALLS analysis of inactivated ZikaV.

Figure 18: Cumulative particle size distribution of Zika NIV.

Figure 19: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 20: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 21: Correlation between JEV antigen content in neutralized inactivated virus (NIV) analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus.

Disclosed herein are downstream processes for purifying Zika virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for

propagation on a particular cell substrate or for a partially / fully cell substrate adapted Zika virus particle.

Aspects of the invention provide processes for the purification of infectious Zika virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the content of residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL.

In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the detection limit of HPLC, in particular below the detection limit in the final drug substance. In some embodiments, the PS content is tested by HPLC or size exclusion chromatography (SEC). For example, HPLC is validated for PS determination in JEV sucrose gradient pool samples as a routine release assay and is very sensitive (i.e., LOQ 3 µg/mL; LOD 1 µg/mL). In the current invention, PS content in in Zika virus DS samples was <LOD. In one embodiment, the HPLC assessment of PS

content can be performed on a Superdex Peptide 10/300GL column (GE: 17-5176-01) using 30% Acetonitrile, 0,1% Trifluoroacetic acid as solvent with a flow rate of 0,6 ml/min at 25°C and detection at 214 nm. A more sensitive method of measurement for residual protamine in a purified virus preparation is mass spectrometry (MS). In some embodiments, the residual PS levels in a Zika virus preparation are tested by MS or other such highly sensitive method, e.g. nuclear magnetic resonance (NMR). With this method, residual PS, as well as fragments and/or break-down products of PS, can be detected at trace amounts, such as levels as low as, for example, 10^6, 10^7 or 10^8 molecules per typical sample load. In some embodiments, the PS levels are tested in the sucrose gradient pool. In some embodiments, the PS levels are tested in the drug product. In some embodiments, the PS levels are tested in the drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of about 100 kDa.

The process according to the current invention may also comprise the use of a sucrose gradient, preferably an optimized sucrose gradient. The sucrose gradient is preferably optimized for the removal of protamine sulfate, also for the removal of immature viral particles or other viral particles which are non-infectious or host cell proteins or nucleic acids (DNA, RNA, mRNA, etc) or other host cell debris. In the current invention the optimized sucrose gradient comprises at least two, at least three, at least four layers of sucrose solutions with different densities. In one embodiment, the virus preparation to be purified is provided in a sucrose solution which has a density of about 8%, about 9%, about 10%, about 11%, about 12% sucrose (w/w), preferably about 10%. In one embodiment, one sucrose solution in the gradient has a density of about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55% sucrose (w/w),

preferably about 50%. In one embodiment, one sucrose solution in the gradient has a density of about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40% sucrose (w/w), preferably about 35%. In one embodiment, one sucrose solution in the gradient has a density of about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20% sucrose (w/w), preferably about 15% sucrose. In a preferred embodiment, the sucrose gradient comprises three layers of sucrose solutions of about 50%, about 35% and about 15% (w/w) sucrose and the virus composition to be purified is contained in about 10% (w/w) sucrose. Because the invention provided for means to not only test for host cell DNA but also immature viral particles, the skilled person in the art is able to more precisely optimize the sucrose gradient for most efficient purification and include additional tools such as PRNT assay to monitor purification success.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may be optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the Zika virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In preferred embodiments, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian

cell line. In some embodiments, said cell line is an EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the Zika virus particles obtainable by any of the processes described herein for treating and/or preventing (i.e. protecting from) a viral infection. In a preferred embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on the average for their age, sex, and ethnicity. This is a result of the brain failing to undergo proper

embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has an RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the process of the invention results in an enrichment of infectious Zika virus particles from the crude harvest comprising infectious Zika virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially at least 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

A unique aspect of the current invention is the realization that know-how related to the vaccine design and purification approach used for the Japanese Encephalitis Vaccine (JEV) IXIARO® (see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) may be employed and improved upon in order to expedite the development of a Zika virus vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, was complemented by further significant improvements disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines is their greatly reduced protamine salt (SEQ ID NO: 1) content in the final drug substance facilitated by the development of an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the Vero (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or the plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently

inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation needs to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0.01 MOI (multiplicity of infection) to allow for viral production prior to passaging. In some embodiments, the Vero cells are incubated for at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. In order to minimize the adaption of Zika virus strain to Vero cells, it was found that Vero cells could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days

2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 µm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the Zika virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant", is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA. The

fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in the experimental part. It was surprisingly found that the addition of a virus-containing fraction with 10% (w/w) sucrose to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) resulted in efficient separation of protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, i.e. a Zika virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agents for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using

such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphobetaine. A preferred inactivation is inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of

time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments, the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time. It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

Any of the methods or uses described herein may be for the prevention of a Zika virus infection in a subject. As used herein, the terms “prevent,” “preventing” and “protection from” include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the Zika virus. As used herein, antigen(s), such as an inactivated Zika virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck, trunk, upper arms; headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject has been previously infected with or vaccinated against Dengue virus; i.e., at risk for antibody-dependent enhancement of disease. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus antibodies). In some embodiments, the therapeutically effective amount is sufficient to provide seroprotection in a subject; i.e., to generate sufficient antigen-specific antibodies to prevent/protect

from infection. In some embodiments, seroprotection is conferred on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects. In some embodiments, seroprotection is defined by a reduction in the number of Zika virus plaques by 50% or more in a plaque reduction neutralization test (PRNT) by a 1:10 or higher dilution of sera from a vaccinated subject. In some embodiments, an effective amount of the Zika vaccine is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the

Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)). Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 71). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its entirety). In some embodiments, the adjuvant is IC31®, i.e. $KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccines to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may

have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having,", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Table 1. Overview of process buffers and stock solutions.

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.

C	25 mM Tris, 150 mM NaCl	7.4±0.2	16.5
D	1 M Tris	7.4±0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
K	10 x PBS	7.4±0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4±0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4±0.2	1.3

Table 2. Abbreviations.

°Bx	Degrees Brix = sugar content (w/w) of an aqueous solution*
BSA	Bovine serum albumin
CC700	Capto™ Core 700
CPE	Cytopathic effect
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
ID	Inner diameter
JEV	Japanese Encephalitis virus
LAL	Limulus amebocyte lysate
LDS buffer	Lithium dodecyl sulfate sample loading buffer
LOD	Limit of detection
LOQ	Limit of quantitation
MALLS	Multiangle light scattering
mAU	Milli absorbance units
MS	Mass spectroscopy
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
PD	Process development
PFU	Plaque forming units
p.i.	Post-infection
PS	Protamine sulphate or protamine sulfate
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin
Rms radius	Root mean square radius
rMSB	Research master seed bank
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SGP	Sucrose gradient purified
SDS	Sodium dodecyl sulphate

TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
WFI	Water for injection
ZikaV	Zika virus

*Degrees Brix (°Bx) is the sugar content of an aqueous solution. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. °Bx corresponds to the sucrose content in percent (w/w), e.g., 45 °Bx equals 45 % (w/w) sucrose.

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Table A. Primers for Zika virus sequencing: lower case letters indicate bases not included in ZIKA but containing restriction sites for later cloning when needed (therefore, two Tms provided).

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
1	9320_Zika_PF_1F	SEQ ID NO: 74 ttaggatccGTTGTTGATCTGTGTGAAT	69.9	74.6	707
	9321_Zika_PF_1R	SEQ ID NO: 75 taactcgagCGTACACAACCCAAGTT	69.3	75.6	
2	9322_Zika_PF_2F	SEQ ID NO: 76 ttaggatccTCACTAGACGTGGGAGTG	70	73.9	704
	9323_Zika_PF_2R	SEQ ID NO: 77 taactcgagAAGCCATGTCYGATATTGAT	69.8	73.7	
3	9324_Zika_PF_3F	SEQ ID NO: 78 ttaggatccGCATACAGCATCAGGTG	72.3	74.5	712
	9325_Zika_PF_3R	SEQ ID NO: 79 taactcgagTGTGGAGTTCCGGTGTCT	72	76.4	
4	9326_Zika_PF_4F	SEQ ID NO: 80 ttaggatccGAATAGAGCGAARGTTGAGATA	70.9	74	712
	9327_Zika_PF_4R	SEQ ID NO: 81 taactcgAGTGGTGGGTGATCTTCTTCT	70.5	73.7	
5	9328_Zika_PF_5F	SEQ ID NO: 82 ttaggatcCAGTCACAGTGGAGGTACAGTAC	70.3	75	704
	9329_Zika_PF_5R	SEQ ID NO: 83 taactcgagCRCAGATACCATCTTCCC	71.5	77.3	
6	9330_Zika_PF_6F	SEQ ID NO: 84 ttaggatCCCTTATGTGCTTGGCCTTAG	70.7	72.7	698
	9331_Zika_PF_6R	SEQ ID NO: 85 taactcgagTCTTCAGCCTCCATGTG	70.4	76.9	
7	9332_Zika_PF_7F	SEQ ID NO: 86 ttaggatccAATGCCCACTCAAACATAGA	71.9	75	716
	9333_Zika_PF_7R	SEQ ID NO: 87 taactcgagTCATTCTCTTCTTCAGCCCTT	71	74	
8	9334_Zika_PF_8F	SEQ ID NO: 88 ttaggatccAAGGGTGATCGAGGAAT	70.9	75.2	703
	9335_Zika_PF_8R	SEQ ID NO: 89 taactcgagTTCCCTTCAGAGAGAGGAGC	71.9	73.4	
9	9336_Zika_PF_9F	SEQ ID NO: 90 ttaggatccTCTTTTGCAAACTGCGATC	71.9	75	699
	9337_Zika_PF_9R	SEQ ID NO: 91 taactcgagTCCAGCTGCAAAGGGTAT	71	74.9	
10	9338_Zika_PF_10F	SEQ ID NO: 92 ttaggatccGTGTGGACATGTACATTGA	71.4	75.8	706
	9339_Zika_PF_10R	SEQ ID NO: 93 taactcgagCCCATTGCCATAAAGTC	70.4	75.8	

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
11	9340_Zika_PF_11F	SEQ ID NO: 94 ttaggatccTCATACTGTGGTCCATGGA	71.6	78.1	692
	9341_Zika_PF_11R	SEQ ID NO: 95 taactcgagGCCCATCTCAACCCTTG	74	78	
12	9342_Zika_PF_12F	SEQ ID NO: 96 ttaggatccTAGAGGGCTTCCAGTGC	70.9	74	707
	9343_Zika_PF_12R	SEQ ID NO: 97 taactcgAGATACTCATCTCCAGGTTTGTTG	70.2	72.2	
13	9344_Zika_PF_13F	SEQ ID NO: 98 ttaggatccGAAAACAAAACATCAAGAGTG	70.6	75.4	726
	9345_Zika_PF_13R	SEQ ID NO: 99 taactcgagGAATCTCTCTGTCATGTGTCCT	71.9	75.6	
14	9346_Zika_PF_14F	SEQ ID NO: 100 ttaggatccTTGATGGCACGACCAAC	73.1	75.6	715
	9347_Zika_PF_14R	SEQ ID NO: 101 ttaggatccGTTGTTGATCTGTGTGAAT	70.8	77.9	
15	9348_Zika_PF_15F	SEQ ID NO: 102 taactcgagCAGGTCAATGTCCATTG	71.9	75.4	719
	9349_Zika_PF_15R	SEQ ID NO: 103 ttaggatccTGTTGTGTTCCTATTGCTGGT	73.9	77.2	
16	9350_Zika_PF_16F	SEQ ID NO: 104 taactcgaGTGATCAGRGCCCCAGC	72.3	75.4	703
	9351_Zika_PF_16R	SEQ ID NO: 105 ttaggatccTGCTGCCCAGAAGAGAA	72	76.3	
17	9352_Zika_PF_17F	SEQ ID NO: 106 taactcgaGCACCAACAYGGGTTCTT	73.6	76	705
	9353_Zika_PF_17R	SEQ ID NO: 107 ttaggatcCTCAAGGACGGTGTGGC	72	75.5	
18	9354_Zika_PF_18F	SEQ ID NO: 108 taactcgagCAATGATCTTCATGTTGGG	71.7	75.8	699
	9355_Zika_PF_18R	SEQ ID NO: 109 ttaggatccTATGGGGGAGGACTGGT	71	74.1	
19	9356_Zika_PF_19F	SEQ ID NO: 110 taactcGAGCCCAGAACCTTGGATC	73.3	75.5	711
	9357_Zika_PF_19R	SEQ ID NO: 111 ttaggatcCAGACCCCCAAGAAGGC	71.3	76.9	
20	9358_Zika_PF_20F	SEQ ID NO: 112 taactcgagCCCCTTTGGTCTTGTCT	71.7	75	706
	9359_Zika_PF_20R	SEQ ID NO: 113 ttaggatccAGGAAGGATGTATGCAGATG	71.9	73.9	
21	9360_Zika_PF_21F	SEQ ID NO: 114 taactcgagACATTTGCGCATATGATTTTG	70.4	75.7	709
	9361_Zika_PF_21R	SEQ ID NO: 115 ttaggatccAGGAAGGACACACAAGAGT	71.8	75	
22	9362_Zika_PF_22F	SEQ ID NO: 116 taactcgagACAGGCTGCACAGCTTT	70	79.1	581
	9363_Zika_PF_22R	SEQ ID NO: 117 ttaggatccTCTCTCATAGGGCACAGAC	74.8	81.1	

SEQUENCES

SEQ ID NO: 1
A typical form of protamine
PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 2
KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome

GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTGG
ATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACG
CGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGG
ATGGTCTTGGCAATTCTAGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTG
GGGAAAAAAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGA
AGGAGAAGAAGAGACGGGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGG
TCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACGATGCTGGGGAGGCCATATCTTTTCCAACCACATTG
GGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCT
GGATGAGGGGGTGGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCAT
CACAAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGT
CGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTC
GCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCT
GATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGG
GTTGATATTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTAC
AACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCT
GCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGC
TGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGTCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCG
GGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGT
TAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACC
CTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATG
AATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAAC
TCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGG
AGTCAAGAAGGAGCAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCT
CTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCA
CATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTG
CAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCA
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SEQ ID NO: 3

KU497555.1 Zika virus isolate Brazil-ZKV2015, Brazil, complete genome

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SEQ ID NO: 4
KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome
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SEQ ID NO: 5
KU509998.1 Zika virus strain Haiti/1225/2014, Haiti, complete genome

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SEQ ID NO: 6

KU527068.1 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, complete genome

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SEQ ID NO: 7
KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome
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SEQ ID NO: 8

KU681082.3 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, complete genome

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KU744693.1 Zika virus isolate VE_Ganxian, China, complete genome

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SEQ ID NO: 11
LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome
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SEQ ID NO: 12

AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome

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SEQ ID NO: 13
KJ776791.1, Zika virus strain H/PF/2013 polyprotein gene, complete cds
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TCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTA
CGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTG
ATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCT
TACATTGTCATAGGAGTCGGGGAGAAGAAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTG
AAGCCACTGTGAGAGGTGCCAAGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCT
CAACTCATTGGGCAAGGGCATCCATCAAATTTTTGGAGCAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACA
AATTCTCATTGGAACGTTGCTGATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGG
GGGAGTGTTGATCTTCTTATCCACAGCTGTCTCTGCTGATGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGAT
GCGGTACAGGGGTGTTCGTCTATAACGACGTTGAAGCCTGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGA
TTGGCAGCAGCAGTCAAGCAAGCCTGGGAAGATGGTATCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTG
GAGATCAGTAGAAGGGGAGCTCAACGCAATCCTGGAAGAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAA
AACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGCCTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAAT
CGTACTTCGTCAGAGCAGCAAAGACAAATAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACAT

AGAGCATGGAACAGCTTTCTTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGA
TTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACT
GGATTGAGAGTGAGAAGAATGACACATGGAGGCTGAAGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAA
AGTCCCACACATTGTGGACAGATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCAT
CACAATACCAGAGAGGGCTACAGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAAT
GCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAG
GGTGATCGAGGAATGGTGCTGCAGGGAGTGCACAATGCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGA
ATGGAGATAAGGCCCAGGAAAGAACCAGAAAGTAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGG
ATCACTTCTCCCTTGGAGTGCTTGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATC
ATAAGCACATCGATGGCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTT
GATGGGTGCCACCTTCGCGGAAATGAACACTGGAGGAGATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGA
CCAGCGTTGCTGGTATCTTTCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTT
TGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGATGGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAG
CGATGGTTGTTCCACGCACTGATAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTG
TGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAA
CTTACCATTTGTCATGGCCCTGGGACTAACCGCTGTGAGGCTGGTCGACCCCATCAACGTGGTGGGACTGCTGTTGCTCAC
AAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTC
GCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGA
GTGTGGACATGTACATTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCT
CGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAG
GTGGTCCTGATGACCATCTGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGAC
TGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTA
CAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGT
GGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATC
TGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCC
CGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCT
GGATTACCCAGCAGGAACTTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTC
GTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGC
CTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAA
ATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGA
AGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAAT
GTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCAC
TTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCAT
GACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAG
AGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAAC
GGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAAACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGT
TCCAGAAAACAAAACATCAAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGAC
CGTGTCATAGATTCCAGGAGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGT
CACACATGCCAGCGCTGCCCAGAGGAGGGGGCGCATAGGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGA
GGTGGGTGCGCAGAGACTGACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGA
TGGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAG
CAAAGGAAGACCTTTGTGGAACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAAT
AACCTACACAGATAGAAGATGGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTG
TGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGA
AGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACA
CATGACAGAGAGATTCCAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAA
GCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTT
TTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATG
TGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAG
CCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTAC
CGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAAC
CATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCA
GCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGT
ATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC

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CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGC
GTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACAC
AATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGT
CGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGA
ACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTA
CACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGG
CCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCC
CGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTG
GAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCA
CCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTA
TGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTG
ACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTT
GAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCA
GCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCG
AAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGA
AATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCAT
TGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGG
GCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAA
ACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAG
GAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGA
AAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATT
AGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGT
GGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGA
AACAAGGGGAATTTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGA
AGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGAGAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACA
AAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCAT
TGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGA
CATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGC
AACTCATTCGGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGAC
CAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATT
GATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAAACCCT
CAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCC
ATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGG
AGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCA
ATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAATGG
ATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACCACATGGAAGACAAGACCCCA
GTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGA
CTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTC
AGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTA
TAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACC
CCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGAT
CAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAG

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13 or 72. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13 or 72.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 14
isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 15
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
RDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 16
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXXXXXXXNR
AEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 17
ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMXXXXXGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 18
ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF

EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 19
ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 20
isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 21
isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 22
isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVE
FKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 23
MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 24

LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 25
isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 26
ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 27
AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 28
MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 29
MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH

AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 30
KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 31
isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 32
CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 33
Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 34
isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 35
KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 36
KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 37
isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 38
BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 39
MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 40
KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 41
Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 42
KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 43
isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 44
isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 45
PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 46
BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 47
H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 48
PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 49
KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 50
ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 51
103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 52
isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 53
KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 54
isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 55
isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 56
isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 57
isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 58
isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 59
isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNGTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 60
isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 61
ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 62
KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 63
isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 64
isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCPTQGEAYLD
KQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENR
AKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFK
DAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVE
VQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 65
isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 66
KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN

RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 67
isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 68
ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E.
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCPTQGEPSL
DKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 69
MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 70
5'-(dIdC)$_{13}$-3'
dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC

SEQ ID NO: 71
KLK peptide
KLKLLLLLKLK

SEQ ID NO: 72
ZIKV Sequence H/PF/2013 as sequenced
CAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTT
TCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTG
AGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCT
AGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAAAAGAGGCTA
TGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGAAGGAGAAGAAGAGAC
GAGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAG
TGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTT
ATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAA

CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCACAAAAAAGGTGAAGC
ACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGTCGCAAACCTGGTTGGAAT
CAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCC
ATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGC
ATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAAC
ATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTACAACAACAGTCAGCAACAT
GGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCGGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAA
GCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTG
GACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCC
AGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGACACAGGACATG
AAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACCCTGGGGGGTTTTGGAAG
CCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATGAATAACAAGCACTGGTT
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SEQ ID NO: 73
AHZ13508.1, Zika virus polyprotein from Polynesian outbreak (H/PF/2013)
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GSAKLRWLVERGYLQPYGKVIDLGCGRGGWSYYAATIRKVQEVKGYTKGGPGHEEPMLVQSYGWNIVRLKSGVDVFHMAAE
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9355_Zika_PF_18R
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SEQ ID NO: 111
9357_Zika_PF_19R

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SEQ ID NO: 112
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SEQ ID NO: 113
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SEQ ID NO: 114
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SEQ ID NO: 116
9362_Zika_PF_22F
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SEQ ID NO: 117
9363_Zika_PF_22R
ttaggatccTCTCTCATAGGGCACAGAC

In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NOs: 14-69 or 72. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69 or 72.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. MoI. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Dr., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977 and Altschul et al., J. MoI. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were adapted to ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 9A and B). Briefly, the unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Zika Virus. As shown in Figure 10, non-infectious virus particle aggregates, HCP and other LMW impurities were removed by PS precipitation as shown by removal of aggregate shoulder in SEC-HPLC and no loss of infectious virus titer by PS treatment. Further optimization of the Zika purification protocol is provided below.

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):

- 5% CO_2, 35°C, MEM+2mM L-Glutamine + 10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01
- Virus Production without serum
- 5% CO_2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (accession number KJ776791.1, also SEQ ID NO: 13 herein) from French Polynesia. A sample was obtained from the European Virus Archive (EVAg; Ref-SKU: 001v-EVA1545). Based on this material, a research master seed bank (rMSB) was prepared on Vero cells as the cell substrate and the genomic sequence was checked by sequencing. Because the genomic sequence at the 5´and 3´flanking sequences of Zika virus strain H/PF/2013 was unknown, primers for sequencing were designed in those regions based on other Zika virus strains whereas the internal primers were designed from the published sequence (SEQ ID NOs: 74 to 117, see also Table A). The sequence obtained from the rMSB by use of these primers is provided by SEQ ID NO: 72. There was 100% overlap of the sequence with the published sequence of Zika Virus Strain H/PF/2013 (SEQ ID NO: 13). However, we sequenced additional regions 5´ (an additional 40 bp) and 3' (an additional 160 bp) represented in SEQ ID NO: 72. In a preferred embodiment, the Zika virus of the invention comprises SEQ ID NO: 72. The genomic RNA is somewhat longer than the sequence according to SEQ ID NO: 72 (perhaps an additional 200 bp). Additionally, a Zika virus adapted to a host cell such as e.g. Vero cells may be expected to contain one or more mutations. For these reasons, the Zika virus of the current invention comprises the sequence of SEQ ID NO: 72 or, preferably, a sequence with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 72. Furthermore, because the viral genome is likely to contain even further flanking regions to SEQ ID NO: 72; in one embodiment, the Zika virus of the invention contains the sequence of SEQ ID NO: 72 and optionally further comprises extensions at the 5' and/or 3' ends of at least 10, at least 20, at least 30, at least 40, at least 50, at least

60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120 or at least 130 nucleotides. In a preferred embodiment, the Zika virus comprises at least the coding sequence for the entire polyprotein of Zika Virus Strain H/PF/2013 of the invention i.e. the amino acid sequence of SEQ ID NO: 73 or a polyprotein with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 73. Furthermore, the Zika virus comprises at least the coding sequence for the E-protein of Zika Virus Strain H/PF/2013 of the invention SEQ ID NO: 47 or an E-protein thereof with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 47.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in a standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and to reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles were infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200 mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significantly higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with the day 3 harvest, the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest.

Table 3: The calculated titers per plaque assay are summarized in the list below.

	Log 10 PFU/mL
sample day2	7.02
harvest day 3	6.66
harvest day 5	6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 15. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22°C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 11 for SEC-HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 20 for SEC-HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 21). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

Samples of individual 30x concentrated harvests days 2, 3, 5 and 7 were analysed before (Figure 11) and after PS (Figure 12) treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 4. The virus peak recovery by SEC-HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 4: ZikaV recovery by SEC-HPLC and plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x + PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x + PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles. Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus particles from residual contaminants as demonstrated for ChikV (Fig. 15D). The optimal volumes of

the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 5.

Table 5: Individual layers/volumes for a centrifugation in bottle.

Solution	**Volume (mL)**
PS treated harvest in 10% sucrose (L)	40
15% sucrose (J)	15
35% sucrose (I)	15
50% sucrose (H)	20
Total volume	90

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 14. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled rotor. (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers.) The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no brake/deceleration activated. In case a different centrifuge system with a different rotor is used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in

position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle (see Figure 14).

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL) and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 14. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51 mL in a PETG container. A volume of 1% formaldehyde (10,000 ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200 ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25ºC) for a minimum of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criterion for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution was monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152 µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. Briefly, a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 21.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gently mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 6).

Table 6: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	**JEV**	**Zika**
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2 µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565.

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 16. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 17) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6 nm and ~49 nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 18).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Table 7: Active ZikaV pfus were quantified by plaque assay throughout the process.

Sample	**Pfu/mL**
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$

Harvest day 5 (filtered)	$1.5x10^8$
Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD
Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2 mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly, the Benzonase treated samples did not yield any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell protein was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well was seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5% CO_2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicate. The target viruses (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were diluted to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were incubated at 35°C with 5% CO_2 for 1 hour. The cell culture medium was aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plates were rocked back and forth 5 times before incubating for 2 hours at 35°C with 5% CO_2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5% CO_2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 19 and 20, respectively). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Another neutralization assay was performed using the microneutralization assay as described by Larocca, et al. (2016, Nature doi:10.1038/nature18952). It was found that the inactivated Zika virus of the current invention had an MN50 (microneutralization) titer of 90 at 1 µg of inactivated purified virus.

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples

are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, http://dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on Vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV vaccine. The neutralization of both the Asian

(H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

The in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, supra.). Inactivated viruses are among the safest vaccines and especially preferred for deliver to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

A4. The vaccine of any one of A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of A1-A9, further comprising an adjuvant.

A11. The vaccine of A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

A15. The vaccine of any one of A1-A14, further comprising one or more pharmaceutically acceptable excipient.

A16. The vaccine of any one of A1-A15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 µg/mL, especially below 100 ng/mL.

A17. The vaccine of A16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

B1. A kit comprising a Zika virus vaccine of any one of A1-A15.

B2. The kit of B1, further comprising a second vaccine.

B3. The kit of B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of A1-A15 to a subject in need thereof.

C2. The method of C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of A1-A15 for the treatment and/or prevention of a Zika virus infection.

E2. The use of E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment or prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of A1-A15.

F2. The pharmaceutical composition of F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

G1. Use of an optimized sucrose gradient centrifugation for removal of protamine sulphate from purified infectious Zika virus particles.

G2. The use according to G1, wherein said optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three further layers of sucrose solutions with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G3. A process of purification of infectious Zika virus particles, comprising the steps of:

a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

G4. The process of G3, wherein said optimized sucrose density gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G5. The process of any one of G3 to G4, additionally comprising a further purification step of: (d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

G6. The process of any of G3 to G5, wherein the residual host cell DNA content of the Zika virus preparation (c) is less than 10 ng/mL and the residual host cell protein content of the final virus preparation (c) is less than 100 ng/mL.

G7. The process of any of G3 to G6, wherein said crude harvest (a) comprising Zika virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

G8. The process of G7, wherein the one or more pre-purification step(s) comprises

a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or
b) digestion of host cell genomic DNA by enzymatic treatment; and/or
c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

G9. The process of any one of G3 to G8, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

G10. The process of any one of G3 to G9, wherein the enrichment of infectious Zika virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

G11. The process of any one of G7 to G10, wherein the one or more pre-purification step(s) prior to step (b) of any of G8 to G11 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

G12. The process of any one of G3 to G11, wherein the residual impurity of the Zika virus preparation (c) is less than 10%.

G13. The process of any one of G3 to G12, wherein the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

G14. The process of G13, wherein said cell line is a Vero cell line.

G15. The process of any one of G3 to G14, wherein said infectious Zika virus particle is an infectious virus particle that is a live virus, a live attenuated virus, a chimeric virus, a modified live virus, or a recombinant live virus.

G16. The process of any one of G3 to G15, wherein said Zika virus is preferably a strain of the Asian lineage.

G17. The process of any one of G3 to G16, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

G18. Use of the process according to any one of G3 to G17 for manufacturing a composition for immunization against a virus infection.

G19. The use according to G18, wherein said virus infection is an infection caused by a Zika virus.

Q1. A process of purification of infectious Zika virus particles, comprising the steps of:
(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

Q2. The process of Q2, wherein the virus particles are from Zika virus.

Q3. The process of Q1 or Q2, additionally comprising the step of:

(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

Q4. The process of any of Q1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

Q5. The process of any of Q1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

Q6. The process of Q5, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

Q7. The process of any one of Q1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

Q8. The process of any one of Q1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

Q9. The process of any one of Q5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of Q5 to 8 is performed using a filter having a pore size equal to or less than 1 µm, preferably 0.2 µm.

Q10. The process of any one of Q1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

Q11. The process of any one of Q1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

Q12. The process of Q11, wherein said cell line is a Vero cell line.

Q13. The process of any one of Q1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

Q14. The process of any one of Q1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

Q15. The process of any one of Q1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

Q16. Use of the process according to any one of Q1 to 15 for manufacturing a composition for immunization against a virus infection.

Q17. The use according to Q16, wherein the composition for immunization against a virus infection is an infection caused by Zika virus.

Q18. A composition comprising the virus particles obtainable or obtained by the process of any one of Q1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

Q19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

Q20. The Zika virus vaccine of Q19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

Q21. The vaccine of Q19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

Q22. The vaccine of any one of Q19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69 or 72, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 or 72 and able to pack a virulent Zika virus.

Q23. The vaccine of any one of Q19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

Q24. The vaccine of Q23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

Q25. The vaccine of Q24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

Q26. The vaccine of any one of Q19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

Q27. The vaccine of Q26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

Q28. The vaccine of Q27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

Q29. The vaccine of Q28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

Q30. The vaccine of any one of Q27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

Q31. The vaccine of any one of Q19 to 30, further comprising an adjuvant.

Q32. The vaccine of Q31, wherein the adjuvant is an aluminum salt adjuvant.

Q33. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

Q34. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

Q35. The vaccine of any one of Q19 to 34, further comprising one or more pharmaceutically acceptable excipient.

R1. Use of protamine, preferably a protamine salt, to separate infectious and non-infectious Zika virus particles, host cell proteins and/or undefined low molecular weight materials.

R2. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b), wherein the enrichment of infectious virus particles in the virus preparation (b) relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R3. The use of R1 or the process of R2, wherein the virus particles are from Zika virus.

R4. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by one or more size exclusion methods such as (i) a sucrose density gradient centrifugation, (ii) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles, and/or (iii) size exclusion chromatography to obtain a virus preparation (c) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (c) is less than 100 ng/mL and the residual host cell protein and the residual aggregates of infectious virus particles of the final virus preparation (c) is less than 1 µg/mL.

R5. The process of R4, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

R6. The process of any of R2 to 5, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

R7. The process of R6, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

R8. The process of any one of R2 to 7, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.

R9. The process of any one of R2 to 8, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R10. The process of any one of R6 to 9, wherein the one or more pre-purification step(s) prior to step (b) of any of R6 to 9 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

R11. The process of any one of R2 to 10, wherein the residual impurity of the virus preparation (c) is less than 10%.

R12. The process of any one of R2 to 11, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

R13. The process of R12, wherein said cell line is a Vero cell line.

R14. The process of any one of R2 to 13, wherein the Zika virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

R15. The process of any one of R2 to 14, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

R16. The process of any one of R2 to 15, wherein said process resulting in final virus preparation (c) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

R17. Use of the process according to any one of R1 to 16 for manufacturing a composition for immunization against a virus infection.

R18. The use according to R17, wherein the composition for immunization against a virus infection is an infection caused by a Zika virus.

R19. A composition comprising the virus particles obtainable or obtained by the process of any one of R2 to 16 for treating and/or preventing an infection.

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Document code: WFEE

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<151> 2016-06-23

<150> EP 16176025.1
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[x] I hereby appoint Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 23628

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter to:

[x] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Valneva Austria GmbH

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[x] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	28 Dec. 2014
Name	Adrian Spillmann, Ph.D., LL.M.		
Title	Head of Corporate Intellectual Property – Valneva Austria GmbH		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[ ] Total of 1 forms are submitted

Adrian SPILLMANN, Ph.D
Global Head IP - Intellectual Property
Valneva Austria GmbH
A-1030 Wien, Campus Vienna Biocenter 3
Tel.: +43-1-20620-1456
Fax: +43-1-20620-81456

3409626.1

Electronic Acknowledgement Receipt

EFS ID:	33655350
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Customer Number:	23628
Filer:	John R. Van Amsterdam/Janet O'Connor
Filer Authorized By:	John R. Van Amsterdam
Attorney Docket Number:	I0422.70125US00
Receipt Date:	07-SEP-2018
Filing Date:	
Time Stamp:	12:10:57
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	I042270125US00-TRN-JRV.pdf	26359 31a0f81888d710f5fabf97f0ae398267fc8007a2	no	1
Warnings:		453			

Information:					
2	Oath or Declaration filed	I042270125US00-DEC-JRV.pdf	1116134 2c78416d1e1129216b9368ee86ccc2a834c388bd	no	2
Warnings:					
Information:					
3	Power of Attorney	I042270125US00-POA-JRV.pdf	1325937 c18817fb1b78f18f7326d8fbf0dbb4e47608040c	no	1
Warnings:					
Information:					
			Total Files Size (in bytes):	2468430	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [x] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

  Declaration

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/John R. Van Amsterdam/		
Printed name	John R. Van Amsterdam		
Date	September 7, 2018	Reg. No.	40,212

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: September 7, 2018 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor

DECLARATION (37 C.F.R. § 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 C.F.R. § 1.76)

Title of Invention	ZIKA VIRUS VACCINE

As the below named inventor, I declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/EP2016/082664 filed on December 23, 2016 Assigned U.S. Serial No. 16/063,007

The above-identified application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above-identified application, including the claims.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. § 1.56.

All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

I hereby acknowledge that any willful false statement and the like made in this declaration is punishable by fine or imprisonment of not more than five (5) years, or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the application or any patent issuing thereon.

LEGAL NAME OF SOLE OR FIRST INVENTOR

Inventor one: Jana Barbero Calzado Date: 2 Aug 2018

Signature:

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor two: Mario Nebenführ Date: 03 AUG 2018

Signature:

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor three: Robert Schlegl Date: 3-Aug-2018

Signature:

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor four: Michael Weber Date: 10-Aug-2018

Signature:

☒ Additional inventors are being named on 1 Additional form(s) attached hereto.

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1
LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any	
Inventor five: Jürgen Heindl-Wruss	Date: 06-Apr-2019
Signature:	

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/063,007	06/15/2018		2120	I0422.70125US00	17	1

CONFIRMATION NO. 4503

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

FILING RECEIPT

OC000000102436080

Date Mailed: 09/21/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Jana BARBERO CALZADO, Vienna, AUSTRIA;
Mario NEBENFÜHR, Vienna, AUSTRIA;
Robert SCHLGEL, Siegenfeld, AUSTRIA;
Michael WEBER, Vienna, AUSTRIA;
Jürgen WRUSS, Vienna, AUSTRIA;

Applicant(s)

VALNEVA AUSTRIA GMBH, Vienna, AUSTRIA;

Assignment For Published Patent Application

Valneva Austria GmbH, Vienna, AUSTRIA

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/EP2016/082664 12/23/2016

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)
EUROPEAN PATENT OFFICE (EPO) 16182845.4 08/04/2016
EUROPEAN PATENT OFFICE (EPO) 16176049.1 06/23/2016
EUROPEAN PATENT OFFICE (EPO) 16176025.1 06/23/2016
EUROPEAN PATENT OFFICE (EPO) 16161068.8 03/18/2016
EUROPEAN PATENT OFFICE (EPO) 15202585.4 12/23/2015

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/19/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/063,007**

Projected Publication Date: 12/27/2018

Non-Publication Request: No

Early Publication Request: No

Title

ZIKA VIRUS VACCINE

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/063,007

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	18 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							820
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2800

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
16/063,007	Jana BARBERO CALZADO	I0422.70125US00

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

INTERNATIONAL APPLICATION NO.	
PCT/EP2016/082664	
I.A. FILING DATE	PRIORITY DATE
12/23/2016	12/23/2015

CONFIRMATION NO. 4503
371 FORMALITIES LETTER



Date Mailed: 09/21/2018

Notification of Insufficiency Under 37 CFR 1.492 and/or 1.495 (DO/EO/US)

The following items have been submitted by the applicant or the International Bureau to the United States Patent and Trademark Office as a Designated / Elected Office (37 CFR 1.495).

- Priority Document
- Copy of the International Application filed on 06/15/2018
- Copy of the International Search Report filed on 06/15/2018
- Preliminary Amendments filed on 06/15/2018
- Sequence Listing txt file (EFS or physical medium) filed on 06/15/2018
- Inventor's Oath or Declaration filed on 09/07/2018
- U.S. Basic National Fees filed on 06/15/2018
- Authorize Access to Search Results filed on 06/15/2018
- Priority Documents filed on 06/15/2018
- Power of Attorney filed on 09/07/2018
- Specification filed on 06/15/2018
- Claims filed on 06/15/2018
- Abstracts filed on 06/15/2018
- Drawings filed on 06/15/2018
- Authorization to Permit Access filed on 06/15/2018
- Application Data Sheet (37 CFR 1.76) filed on 06/15/2018

The following items **MUST** be furnished within the period set forth below:

- Additional claim fees of $**820** as a undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**820** for an Undiscounted Entity:

Total additional claim fee(s) for this application is $**820**
- $**820** for multiple dependent claim surcharge.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

SHAKEEL AHMED

Telephone: (703) 756-1423

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
16063007	

Applicant(s) Jana BARBERO CALZADO

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
2		1				
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Total Indep	1		0		0	
Total Depend	17	↵	0	↵	0	↵
Total Claims	18		0		0	

CLAIMS	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
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UNITED STATES PATENT AND TRADEMARK OFFICE

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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00

CONFIRMATION NO. 4503

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

PUBLICATION NOTICE

OC000000104715805

Title:ZIKA VIRUS VACCINE

Publication No.US-2018-0371027-A1
Publication Date:12/27/2018

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	Not Yet Assigned
Art Unit:	Not Yet Assigned

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: January 24, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

REQUEST FOR CORRECTED PUBLICATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant in the above-referenced application respectfully requests correction of the corresponding published application, US 2018-0371027-A1, as noted below. This application was published on December 27, 2018 and accordingly this request is timely.

The published application includes a material error in claim 14. Specifically, the peptide sequence KLKL5KLK (SEQ ID NO: 71) is incorrectly written with a "S" in place of the "5": KLKLSKLK. This error in a sequence recited in claim 14 is material at least because the sequence as written in the claim (KLKLSKLK) does not match the correct sequence in claim 14 as filed (KLKL5KLK), nor the sequence provided at SEQ ID NO: 71.

Therefore, correction of the publication to recite the correct sequence in claim 14, KLKL5KLK (SEQ ID NO: 71), is respectfully requested.

Applicant believes no fee is due with this request. However, if a fee is due with this request, please charge Deposit Account No. 23/2825 under Docket No. I0422.70125US00 from which the undersigned is authorized to draw.

Dated: January 24, 2019

Respectfully submitted,

By: /John R. Van Amsterdam/
John R. Van Amsterdam
Registration No.: 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Electronic Acknowledgement Receipt

EFS ID:	34944447
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
Filer:	John R. Van Amsterdam/Janet O'Connor
Filer Authorized By:	John R. Van Amsterdam
Attorney Docket Number:	I0422.70125US00
Receipt Date:	24-JAN-2019
Filing Date:	15-JUN-2018
Time Stamp:	10:18:46
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	I042270125US00-TRN-JRV.pdf	26472 fec816f904c84c1ba4746fa7fa9d6d585fcc772e	no	1
Warnings:		469			

Information:					
2	Request/Corrected Patent Application Publications	I042270125US00-RCPUB-JRV.pdf	25434 a268e4003b3cf578f1190811aeff7ead71c3a6f3	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	51906	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) (please Identify below):

  Request for Corrected Publication

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/John R. Van Amsterdam/		
Printed name	John R. Van Amsterdam		
Date	January 24, 2019	Reg. No.	40,212

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: January 24, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	Not Yet Assigned
Art Unit:	Not Yet Assigned

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 21, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

SECOND PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

FEE TRANSMITTAL

Complete if known	
Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Practitioner Docket No.	I0422.70125US00

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)2,200.00

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below

☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

☐ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
20 - or HP =	0 x		=

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
1 - or HP =	0 x		=

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
- 100 =	/50 =	(round **up** to a whole number) x		=

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1254 Extension for response within fourth month	2,200.00

SUBMITTED BY

Signature	/John R. Van Amsterdam/	Registration No. (Attorney/Agent)	40,212	Telephone	617.646.8000
Name (Print/Type)	John R. Van Amsterdam			Date	March 21, 2019

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 21, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

Electronic Patent Application Fee Transmittal

Application Number:	16063007
Filing Date:	15-Jun-2018
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Filer:	John R. Van Amsterdam/Janet O'Connor
Attorney Docket Number:	I0422.70125US00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 4 months with $0 paid	1254	1	2200	2200
Miscellaneous:				
			Total in USD ($)	**2200**

Electronic Acknowledgement Receipt

EFS ID:	35474150
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
Filer:	John R. Van Amsterdam/Janet O'Connor
Filer Authorized By:	John R. Van Amsterdam
Attorney Docket Number:	I0422.70125US00
Receipt Date:	20-MAR-2019
Filing Date:	15-JUN-2018
Time Stamp:	09:43:20
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2200
RAM confirmation Number	032019INTEFSW09434600
Deposit Account	232825
Authorized User	Janet O'Connor

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	I042270125US00-TRN-JRV.pdf	27380 e7b758af8a7f9ada9172c834e1b6f3b2fc0e2bac	no	1
Warnings:					
Information:					
2	Extension of Time	I042270125US00-EXT-JRV.pdf	24403 1917c993b0aae7654b1d26d5033a24f9a71f09ae	no	1
Warnings:					
Information:					
3	Applicant Response to Pre-Exam Formalities Notice	I042270125US00-RTNI-JRV.pdf	24443 7bb5a804f155963193e4a49b24ac548b45f314db	no	1
Warnings:					
Information:					
4	Application Data Sheet	I042270125US00-CADS-JRV.pdf	25133 30f315d7cb9201b47480cc1acebd89a4c61a0c10	no	6
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
5		I042270125US00-AMN-JRV.pdf	43659 764534fb4c26e810b97615dbacf194e025f03bba	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Applicant Arguments/Remarks Made in an Amendment	5	5
	Claims	2	4

	Preliminary Amendment		1	1	
Warnings:					
Information:					
6	Fee Worksheet (SB06)	I042270125US00-FEE-JRV.pdf	31755 60e8bdb40325538676260f4fae570df396ba00b5	no	1
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	30638 59e31ce187cba9e1e9ac019ce92d59a276a61c9c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	207411		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	16/063,007-Conf. #4503
		Filing Date	June 15, 2018
		First Named Inventor	Jana Barbero Calzado
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
Total Number of Pages in This Submission		Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

[X] Fee Transmittal Form	[ ] Drawing(s)	[ ] After Allowance Communication to TC
[ ] Fee Attached	[ ] Licensing-related Papers	[ ] Appeal Communication to Board of Appeals and Interferences
[X] Amendment/Reply	[ ] Petition	[ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
[ ] After Final	[ ] Petition to Convert to a Provisional Application	[ ] Proprietary Information
[ ] Affidavits/declaration(s)	[ ] Power of Attorney, Revocation Change of Correspondence Address	[ ] Status Letter
[X] Extension of Time Request	[ ] Terminal Disclaimer	[X] Other Enclosure(s) (please Identify below): • Response to Notification of Insufficiency • Corrected Application Data Sheet
[ ] Express Abandonment Request	[ ] Request for Refund	
[ ] Information Disclosure Statement	[ ] CD, Number of CD(s) _____	
[ ] Certified Copy of Priority Document(s)	[ ] Landscape Table on CD	
[ ] Reply to Missing Parts/ Incomplete Application	Remarks	
[ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/John R. Van Amsterdam/		
Printed name	John R. Van Amsterdam		
Date	March 21, 2019	Reg. No.	40,212

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 21, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

PTO/AIA/22 (03-13)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) I0422.70125US00
Application Number 16/063,007-Conf. #4503	Filed June 15, 2018
For ZIKA VIRUS VACCINE	
Art Unit Not Yet Assigned	Examiner Not Yet Assigned

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☐ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☒ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$ 2,200
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

☒ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☒ attorney or agent of record. Registration number 40,212 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/John R. Van Amsterdam/	March 21, 2019
Signature	Date
John R. Van Amsterdam	617.646.8000
Typed or printed name	Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 21, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	Not Yet Assigned
Art Unit:	Not Yet Assigned

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 21, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

RESPONSE TO NOTIFICATION OF INSUFFICIENCY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Notification of Insufficiency mailed September 21, 2018, Applicant respectfully submits a Preliminary Amendment, petition for a four month extension of time and fees (as shown on accompanying Fee Transmittal).

Please charge our Credit Card in the amount of $2,200.00 covering the fee set forth in 37 C.F.R. §1.17(a)(4). The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to Deposit Account No. 23/2825, under Docket No. I0422.70125US00.

Dated: March 21, 2019

Respectfully submitted,

By: /John R. Van Amsterdam/
John R. Van Amsterdam
Registration No.: 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Corrected Application Data Sheet

Inventor Information

Inventor Number::	1
Given Name::	Jana
Family Name::	Barbero Calzado
City of Residence::	Vienna
Country of Residence::	Austria
Street of mailing address::	Mittersteig 20/21
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1050

Inventor Number::	2
Given Name::	Mario
Family Name::	Nebenführ
City of Residence::	Vienna
Country of Residence::	Austria
Street of mailing address::	Grimmelshausengasse 12/27
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1030

Inventor Number:: 3

Given Name::	Robert
Family Name::	Schlegl
City of Residence::	Siegenfeld
Country of Residence::	Austria
Street of mailing address::	Rosenthal 63
City of mailing address::	Siegenfeld
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-2500

Inventor Number::	4
Given Name::	Michael
Family Name::	Weber
City of Residence::	Vienna
Country of Residence::	Austria
Street of mailing address::	Rechte Wienzeile 249/15
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1120

Inventor Number::	5
Given Name::	Jürgen
Family Name::	Heindl-Wruss
City of Residence::	Vienna

Country of Residence::	Austria
Street of mailing address::	~~Dampfgasse 25/27/-29~~ Leebgasse 78/22
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1100

Correspondence Information

Correspondence Customer Number::	23628

Application Information

Application Number::	16/063,007
Filing Date::	06/15/18
Application Type::	Regular
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	Yes
Computer Readable Form (CRF)?::	Text File
Title::	ZIKA VIRUS VACCINE
Attorney Docket Number::	I0422.70125US00
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	23
Small Entity?::	No

Petition included?:: No

Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2:: No

Representative Information

Representative Customer Number:: 23628

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::	Prior Appl Status::
This Application	a 371 of international	PCT/EP2016/082664	12/23/16	

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::	DAS Access::
European Patent Office	16182845.4	08/04/16	Yes	
European Patent Office	16176049.1	06/23/16	Yes	
European Patent Office	16176025.1	06/23/16	Yes	
European Patent Office	16161068.8	03/18/16	Yes	
European Patent Office	15202585.4	12/23/15	Yes	

Applicant Information

Applicant Number:: 1

Applicant Type:: Assignee

Organization Name::	Valneva Austria GmbH
Street of mailing address::	Campus Vienna Biocenter 3
	1030 Vienna
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1030

Assignee Information Including Non-Applicant Assignee Information

Assignee Number::	1
Organization Name::	Valneva Austria GmbH
Street of mailing address::	Campus Vienna Biocenter 3
	1030 Vienna
City of mailing address::	Vienna
Country of mailing address::	Austria
Postal or Zip Code of mailing address::	A-1030

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).** This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants. See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/John R. Van Amsterdam/	Date (YYYY-MM-DD)	2019-03-21
Name	John R. Van Amsterdam	Registration Number	40,212

REMARKS

By this Preliminary Amendment, claims 1-17 have been amended and new claims 18-20 have been added. Support for the claim amendments is found throughout the specification and in the claims as originally filed. Support for the new claims is found throughout the specification. No new matter has been added.

Applicant reserves the right to pursue the claims as originally filed in one or more continuing applications.

Dated: March 21, 2019

Respectfully submitted,

By: /John R. Van Amsterdam/

John R. Van Amsterdam
Registration No.: 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

AMENDMENTS TO THE CLAIMS

Please amend the claims as follows. Applicant presents a full set of claims showing markups of the claims with insertions and deletions indicated by underlining and strikethrough text (or double bracketing), respectively.

1. (Currently amended) A Zika virus vaccine comprising an inactivated Zika virus ~~particle~~, wherein ~~said~~ the Zika virus vaccine contains protamine sulphate (PS) or fragments or break-down products of PS at amounts below 1 µg/mL and wherein the vaccine is able to confer seroprotection on at least 70% of subjects that are administered the Zika virus vaccine.

2. (Currently amended) The Zika virus vaccine of claim 1, wherein the Zika virus vaccine ~~particle~~ is able to confer seroprotection on at least [[75%,]] 80%~~, 90%, 95%, 96%, 97%, 98%, or at least 99%~~ of ~~vaccinated~~ subjects that are administered the Zika virus vaccine~~, preferably on at least 80% of subjects~~.

3. (Currently amended) The Zika virus vaccine of claim 1 [[or 2]], wherein the Zika virus ~~particle has a~~ comprises an RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

4. (Currently amended) The Zika virus vaccine of claim 1 ~~any one of claims 1 to 3~~, wherein the Zika virus ~~particle has~~ comprises an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

5. (Currently amended) The Zika virus vaccine of claim 1 ~~any one of claims 1 to 4~~, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

6. (Currently amended) The Zika virus vaccine of claim 5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

7. (Currently amended) The Zika virus vaccine of claim 6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

8. (Currently amended) The Zika virus vaccine of claim 7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

9. (Currently amended) The Zika virus vaccine of claim 5 ~~any one of claims 5 to 8~~, wherein the chemical activation is performed at about +4°C or about +22°C.

10. (Currently amended) The Zika virus vaccine of claim 1 ~~any one of claims 1 to 9~~, further comprising an adjuvant.

11. (Currently amended) The Zika virus vaccine of claim 10, wherein the adjuvant is an aluminum salt adjuvant.

12. (Currently amended) The Zika virus vaccine of claim 11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

13. (Currently amended) The Zika virus vaccine of claim 10 ~~any one of claims 10 to 12~~, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

14. (Currently amended) The Zika virus vaccine of claim 13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

15. (Currently amended) The Zika virus vaccine of claim 1 ~~any one of claims 1 to 14~~, further comprising one or more pharmaceutically acceptable excipients.

16. (Currently amended) The Zika virus vaccine of claim 1 ~~any one of claims 1 to 15~~, wherein the Zika virus vaccine contains protamine sulphate (PS) or fragments or break-down products of PS at amounts ~~too low to detect by HPLC, i.e., below 1 µg/mL, especially~~ below 100 ng/mL.

17. (Currently amended) The Zika virus vaccine of claim 1[[16]], wherein said protamine sulphate (PS) or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

18. (New) The Zika virus vaccine of claim 1, wherein said Zika virus vaccine is able to confer seroprotection on at least 90% of subjects that are administered the Zika virus vaccine.

19. (New) The Zika virus vaccine of claim 1, wherein said Zika virus vaccine is able to confer seroprotection on at least 95% of subjects that are administered the Zika virus vaccine.

20. (New) The Zika virus vaccine of claim 11, wherein said aluminium salt adjuvant is aluminium hydroxide with less than 1.25 parts per billion (ppb) copper (Cu) based on the final pharmaceutical composition comprising the Zika virus.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00

CONFIRMATION NO. 4503

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

37 CFR 1.48(f)
ACKNOWLEDGEMENT LETTER

OC000000106913469

Date Mailed: 03/29/2019

NOTICE OF ACCEPTANCE OF REQUEST UNDER 37 CFR 1.48(f)

This is in response to the applicant's request under 37 CFR 1.48(f) submitted on 03/20/2019.

The request under 37 CFR 1.48(f) to correct the inventorship, to correct or update the name of an inventor, or to correct the order of names of joint inventors is accepted.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sahmed/

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/063,007	06/15/2018	1648	2120	I0422.70125US00	20	1

CONFIRMATION NO. 4503

UPDATED FILING RECEIPT

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

OC000000106913481

Date Mailed: 03/29/2019

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Jana BARBERO CALZADO, Vienna, AUSTRIA;
Mario NEBENFÜHR, Vienna, AUSTRIA;
Robert SCHLGEL, Siegenfeld, AUSTRIA;
Michael WEBER, Vienna, AUSTRIA;
Jürgen HEINDL-WRUSS, Vienna, AUSTRIA;

Applicant(s)

VALNEVA AUSTRIA GMBH, Vienna, AUSTRIA;

Assignment For Published Patent Application

Valneva Austria GmbH, Vienna, AUSTRIA

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/EP2016/082664 12/23/2016

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)
EUROPEAN PATENT OFFICE (EPO) 16182845.4 08/04/2016
EUROPEAN PATENT OFFICE (EPO) 16176049.1 06/23/2016
EUROPEAN PATENT OFFICE (EPO) 16176025.1 06/23/2016
EUROPEAN PATENT OFFICE (EPO) 16161068.8 03/18/2016
EUROPEAN PATENT OFFICE (EPO) 15202585.4 12/23/2015

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/19/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/063,007**
Projected Publication Date: Not Applicable
Non-Publication Request: No
Early Publication Request: No
Title

ZIKA VIRUS VACCINE

Preliminary Class

424

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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UNITED STATES PATENT AND TRADEMARK OFFICE

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www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
16/063,007	Jana BARBERO CALZADO	I0422.70125US00

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

INTERNATIONAL APPLICATION NO.	
PCT/EP2016/082664	
I.A. FILING DATE	PRIORITY DATE
12/23/2016	12/23/2015

CONFIRMATION NO. 4503
371 ACCEPTANCE LETTER

OC000000106913462

Date Mailed: 03/29/2019

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

06/15/2018
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 06/15/2018
- Copy of the International Search Report filed on 06/15/2018
- Preliminary Amendments filed on 06/15/2018
- Sequence Listing txt file (EFS or physical medium) filed on 06/15/2018
- Inventor's Oath or Declaration filed on 09/07/2018
- U.S. Basic National Fees filed on 06/15/2018
- Authorize Access to Search Results filed on 06/15/2018
- Priority Documents filed on 06/15/2018
- Power of Attorney filed on 09/07/2018
- Specification filed on 06/15/2018
- Claims filed on 06/15/2018
- Abstracts filed on 06/15/2018
- Drawings filed on 06/15/2018
- Authorization to Permit Access filed on 06/15/2018
- 37 CFR 1.48(f) request to correct inventor's name filed on 03/20/2019
- Application Data Sheet (37 CFR 1.76) filed on 06/15/2018

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's

oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.495(c).

- The name of the inventor set forth in the inventor's oath or declaration does not correspond to the name of the inventor as established under 37 CFR 1.41(e) or corrected under 37 CFR 1.48 for the inventor listed below. Any request to correct or update the name of an inventor must be made in accordance with the provisions of 37 CFR 1.48(f) and include an ADS in accordance with 37 CFR 1.76 (including markings showing the changes) that identifies each inventor by his or her legal name and the required processing fee: **Jürgen HEINDL-WRUSS**.

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

SHAKEEL AHMED

Telephone: (703) 756-1423

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/063,007

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1980

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

Inventor Declaration

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/John R. Van Amsterdam/		
Printed name	John R. Van Amsterdam		
Date	April 8, 2019	Reg. No.	40,212

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: April 8, 2019 Electronic Signature for Janet S. O'Connor: /Janet S. O'Connor/

DECLARATION (37 C.F.R. § 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 C.F.R. § 1.76)

Title of Invention	ZIKA VIRUS VACCINE

As the below named inventor, I declare that:

This declaration is directed to:

[ ] The attached application, or

[x] United States application or PCT international application number PCT/EP2016/082684 filed on December 23, 2016 Assigned U.S. Serial No. 16/063,007

The above-identified application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above-identified application, including the claims.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. § 1.56.

All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

I hereby acknowledge that any willful false statement and the like made in this declaration is punishable by fine or imprisonment of not more than five (5) years, or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the application or any patent issuing thereon.

LEGAL NAME OF SOLE OR FIRST INVENTOR

Inventor one: Jana Barbero Calzado Date: 02/08/2018

Signature:

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor two: Mario Nebenführ Date: 03/08/2018

Signature:

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor three: Robert Schlegl Date: 3-Aug-2018

Signature:

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor four: Michael Weber Date: 10-Aug-2018

Signature:

[X] Additional inventors are being named on 1 Additional form(s) attached hereto.

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1
LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any	
Inventor five: Jürgen Heindl-Wruss	Date: 06-Apr-2019
Signature:	

Electronic Acknowledgement Receipt

EFS ID:	35651750
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
Filer:	John R. Van Amsterdam/Janet O'Connor
Filer Authorized By:	John R. Van Amsterdam
Attorney Docket Number:	I0422.70125US00
Receipt Date:	08-APR-2019
Filing Date:	15-JUN-2018
Time Stamp:	11:01:00
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	I042270125US00-TRN-JRV.pdf	25917 6f1445ee0e7dd22154071051ba597c9fd6049027	no	1
Warnings:		503			

Information:					
2	Oath or Declaration filed	I042270125US00-INVDEC-JRV.pdf	1082085 7c685ef8bb3a3e73d86f25cf607904446972 1856	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	1108002	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 09/23/2019

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
CHESTNUT, BARRY A	

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
09/23/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 16/063,007	Applicant(s) BARBERO CALZADO et al.	
	Examiner Barry Chestnut	Art Unit 1648	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 03/20/2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-20 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1-20 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Election/Restrictions

Restriction is required under 35 U.S.C. 121 and 372.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

The species are as follows:

Claim 3, drawn to the Zika virus vaccine of claim 1, wherein the Zika virus comprises an RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus. Applicant must elect one SEQ ID NO.

Claim 4, drawn to the Zika virus vaccine of claim 1, wherein the Zika virus comprises an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus. Applicant must elect one SEQ ID NO.

The species lack unity because they are not coextensive with each other. The species are drawn to antigens of a virus or other microorganism, whereby all have biological functions and activities that are not a single general inventive concept.

Applicant is required, in reply to this action, to elect a single species to which the claims shall be restricted if no generic claim is finally held to be allowable. The reply must also identify the claims readable on the elected species, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered non-responsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise require all the limitations of an allowed generic claim. Currently, the following claim(s) are generic:

Claim 1.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**. The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention. Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Barry Chestnut whose telephone number is (571)270-3546. The examiner can normally be reached on M-F 8am-4pm EST. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Janet Andres can be reached on (571) 272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300. Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/BARRY A CHESTNUT/
Primary Examiner, Art Unit 1648

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 09/25/2019

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
CHESTNUT, BARRY A	

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
09/25/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

UNITED STATES PATENT AND TRADEMARK OFFICE

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In re Application of	:	
BARBERO CALZADO et al.	:	
Application No. 16/063,007	:	DECISION ON PETITION
Filed: 15 Jun 2018	:	
For: ZIKA VIRUS VACCINE	:	

This is a decision on the request for correction of patent application publication under 37 CFR 1.221(b), received on January 24, 2019, for the above-identified application.

The request is granted

Applicant requests that the application be republished because the patent application publication contains errors in claim 14.

The instant request identifies material Office mistakes in claim 14.

The corrected patent application publication will be published in due course, unless the patent issues before the application is republished.

Inquiries relating to this matter may be directed to the undersigned at (571) 272-6692. Inquiries concerning the Pre-Grant Publication, or republication, should be directed to the Office of Data Management, Office of Publications, at (571) 272-4200.

/Christopher Bottorff/

Christopher Bottorff
Petitions Examiner
Office of Petitions

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00

CONFIRMATION NO. 4503

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

PUB REQUEST ACCEPTANCE LETTER

OC000000111772638

Date Mailed: 10/10/2019

NOTICE OF ACCEPTANCE OF PUBLICATION REQUEST

The request for voluntary publication, amended publication, early publication, redacted publication, republication, corrected publication or revised publication has been received for this application. The request, including payment of any necessary fee(s), is in compliance with 37 CFR 1.215, 1.217, 1.219 or 1.221.

The projected publication date is 01/16/2020.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/dterry/

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	B. A. Chestnut
Art Unit:	1648

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

RESPONSE TO SPECIES ELECTION REQUIREMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is responsive to the Species Election Requirement set forth in the Office Action mailed September 23, 2019. This paper is filed along with a request for a one month extension of time, thereby extending the period for response to December 23, 2019.

In response to the Species Election Requirement, Applicant hereby provisionally elects the following species:

- SEQ ID NO: 3 as the species of DNA sequence corresponding to the RNA genome of the Zika virus recited in claim 3; and
- SEQ ID NO: 47 as the species of E protein amino acid sequence in claim 4.

Claims 1-20 form a group of claims corresponding to the elected species.

Having made this election, Applicant expressly reserves the right to file one or more continuing applications on the subject matter of the non-elected species, in the event that the additional species are not rejoined and considered in this application by the Examiner.

Applicant's election is made without prejudice. As noted by the Examiner, upon allowance of a generic claim, Applicant will be entitled to consideration of claims to additional species, provided that all claims to each additional species are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 C.F.R. § 1.146.

Applicant believes no fee is due with this response other than the enclosed fee associated with the extension of time. However, if any other fee is due with this response, please charge our Deposit Account No. 23/2825, under Docket No. I0422.70125US00 from which the undersigned is authorized to draw.

Dated: December 23, 2019

Respectfully submitted,

By /Chelsea E. Witte-Garcia/
Chelsea E. Witte-Garcia, Ph.D.
Registration No.: 75,713
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

PTO/AIA/22 (03-13)
Approved for use through 11/30/2020. OMB 0651-0031
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) I0422.70125US00
Application Number 16/063,007-Conf. #4503	Filed June 15, 2018
For ZIKA VIRUS VACCINE	
Art Unit 1648	Examiner B. A. Chestnut

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
[X] One month (37 CFR 1.17(a)(1))	$200	$100	$50	$ 200.00
[ ] Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
[ ] Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
[ ] Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
[ ] Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

[ ] Applicant asserts small entity status. See 37 CFR 1.27.

[ ] Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

[ ] A check in the amount of the fee is enclosed.

[X] Payment by credit card. ~~Form PTO-2038 is attached.~~

[ ] The Director has already been authorized to charge fees in this application to a Deposit Account.

[X] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

[X] Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant.

[X] attorney or agent of record. Registration number 75,713 .

[ ] attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Chelsea E. Witte-Garcia/	December 23, 2019
Signature	Date
Chelsea E. Witte-Garcia, Ph.D.	617.646.8000
Typed or printed name	Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

[ ] * Total of 1 forms are submitted.

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

FEE TRANSMITTAL

Complete if known	
Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Examiner Name	B. A. Chestnut
Art Unit	1648
Practitioner Docket No.	I0422.70125US00

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)200.00

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below

☐ Charge fee(s) indicated below, **except for the filing fee**

☐ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- or HP =	_____	x _____	=	_____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- or HP =	_____	x _____	=	_____

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____	- 100 =	_____	/50 =	_____ (round **up** to a whole number)	x _____	=

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1251 Extension for response within first month	200.00

SUBMITTED BY

Signature	/Chelsea E. Witte-Garcia/	Registration No. (Attorney/Agent)	75,713	Telephone	617.646.8000
Name (Print/Type)	Chelsea E. Witte-Garcia, Ph.D.			Date	December 23, 2019

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

Electronic Patent Application Fee Transmittal

Application Number:	16063007
Filing Date:	15-Jun-2018
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Filer:	Chelsea E. Witte-Garcia/Katelyn Farmer
Attorney Docket Number:	I0422.70125US00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
			Total in USD ($)	**200**

Electronic Acknowledgement Receipt

EFS ID:	38117130
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
Filer:	Chelsea E. Witte-Garcia/Katelyn Farmer
Filer Authorized By:	Chelsea E. Witte-Garcia
Attorney Docket Number:	I0422.70125US00
Receipt Date:	23-DEC-2019
Filing Date:	15-JUN-2018
Time Stamp:	10:37:18
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$200
RAM confirmation Number	E2019BMA38040818
Deposit Account	232825
Authorized User	Katelyn Farmer

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	I042270125US00-TRN-CEW.pdf	26080 eb773213ebf6e5148a51c3af32b6339d49d97d8d	no	1
Warnings:					
Information:					
2	Response to Election / Restriction Filed	I042270125US00-ERRR-CEW.pdf	27257 f452d7837d4e3e09cf90d2d02212901f097bc702	no	2
Warnings:					
Information:					
3	Extension of Time	I042270125US00-EXT-CEW.pdf	24353 8c516acf05aa6bf7718b485078496b803340b37c	no	1
Warnings:					
Information:					
4	Fee Worksheet (SB06)	I042270125US00-FEE-CEW.pdf	32587 ffe0c23d642721afee540bad587dbc580e466705	no	1
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30870 f24c7fede7a3c70118dc3f39920fefbce407fdca	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	141147		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	1648
Examiner Name	B. A. Chestnut
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- [X] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [X] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) (please Identify below):

-Response to Species Election Requirement

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Chelsea E. Witte-Garcia/		
Printed name	Chelsea E. Witte-Garcia, Ph.D.		
Date	December 23, 2019	Reg. No.	75,713

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	1	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
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		8,765,148	B2	Wizel et al.	07-01-2014
		10,086,061	B2	Thomas et al.	10-02-2018
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		2018-0362936	A1	Barbero Calzado et al.	12-20-2018
		2019-0008945	A1	Barbero Calzado et al.	01-10-2019

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		CN	105749268	A	Sinovac Research and Development Co Ltd.	07-13-2016	Y-Abstract
		WO	1999/011762	A1	Cheil Jedang Corporation, et al.	03-11-1999	
		WO	2001/092552	A2	Chiron Corporation	12-06-2001	
		WO	2013/083726	A1	Intercell AG	06-13-2013	
		WO	2016/145149	A1	The United States of America, as represented by the Secretary of the Army, on Behalf of the Walter Reed Army Institute of Research	09-15-2016	
		WO	2017/009873	A1	Bharat Biotech International Limited	01-19-2017	

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
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EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	2	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

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EXAMINER:	DATE CONSIDERED:

[#] EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	3	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

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EXAMINER:	DATE CONSIDERED:

[#] EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	4	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

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		WEAVER, Arrival of Chikungunya Virus in the New World: Prospects for Spread and Impact on Public Health. PLoS Negl Trop Dis. 2014;8(6):e2921. doi:10.1371/journal.pntd.0002921.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
P5706PC00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP2016/082664	23 December 2016 (23-12-2016)	23 December 2015 (23-12-2015)
Applicant		
VALNEVA AUSTRIA GMBH		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [X] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/082664

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/082664

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 C07K14/005
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	B. D. COX ET AL: "Predicting Zika virus structural biology: Challenges and opportunities for intervention", ANTIVIRAL CHEMISTRY & CHEMOTHERAPY., vol. 24, no. 3-4, 1 August 2015 (2015-08-01), pages 118-126, XP055328128, GB ISSN: 0956-3202, DOI: 10.1177/2040206616653873 the whole document ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2017	10/04/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lewis, Birgit

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987, ISSN: 0264-410X [retrieved on 2001-08-14] cited in the application the whole document	1-17
Y	US 8 765 148 B2 (WIZEL BENJAMIN [AT] ET AL) 1 July 2014 (2014-07-01) cited in the application column 1	13,14
X,P	WO 2016/145149 A1 (THE US SECRETARY OF THE ARMY ON BEHALF OF THE WALTER REED ARMY INST OF) 15 September 2016 (2016-09-15) page 5, lines 8-17 paragraphs [0037] - [0040] paragraph [0048] claims 1-19	1-12,15
E	WO 2017/009873 A1 (BHARAT BIOTECH INT LTD) 19 January 2017 (2017-01-19) examples 1, 2,3,5-7	1-12,15
A	US 6 309 650 B1 (KIM HYUN SU ET AL) 30 October 2001 (2001-10-30) cited in the application examples 3-5; tables 5,6	1-17
X,P	CN 105 749 268 A (SINOVAC RES & DEV CO LTD) 13 July 2016 (2016-07-13) the whole document	1-8, 10-12,15
X,P	RAFAEL A. LAROCCA ET AL: "Vaccine protection against Zika virus from Brazil", NATURE, vol. 536, no. 7617, 28 June 2016 (2016-06-28), pages 474-478, XP055314483, United Kingdom ISSN: 0028-0836, DOI: 10.1038/nature18952 cited in the application abstract page 475, right-hand column, last paragraph - page 476, left-hand column, paragraph 1	1-12, 15-17

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/082664

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 8765148 B2	01-07-2014	EP 2536427 A1	26-12-2012
		US 2012308618 A1	06-12-2012
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US 6309650 B1	30-10-2001	AU 748730 B2	13-06-2002
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		NZ 503522 A	29-06-2001
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CN 105749268 A	13-07-2016	NONE	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference P5706PC00	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/EP2016/082664	International filing date *(day/month/year)* 23 December 2016 (23.12.2016)	Priority date *(day/month/year)* 23 December 2015 (23.12.2015)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant VALNEVA AUSTRIA GMBH		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☒	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☒	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 26 June 2018 (26.06.2018)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Nora Lindner e-mail: pct.team5@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2016/082664	23.12.2016	23.12.2015

International Patent Classification (IPC) or both national classification and IPC
INV. A61K39/12 C07K14/005

Applicant
VALNEVA AUSTRIA GMBH

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Lewis, Birgit Telephone No. +49 89 2399-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☐ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

   see separate sheet

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-17
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item II

Priority

The present application claims 5 priorities.

The first priority (23.12.2015) is not valid because patent application EP15202585 does not relate to Zika virus at all.

The second priority is dated 18.03.2016. EP16161068 relates to Zika virus including an inactivated Zika virus but not to a genome of Zika strain H/PF/2013 (SEQ ID NO: 13 of the present application, accession number KJ776791.1). Example 2 discloses virus growth and purification including a concentration step by chilling in ice for 2 hours after adding 0.5-2mg of protamine sulfate and ultracentrifugation on sucrose gradients at 38.000 g for 18 hours followed by filtration through a 0.22 µm filter. Example 3 relates to virus inactivation by formaldehyde (0.018% at 22°c or 4°C). Example 4 tests the immunogenicity of the virus with aluminium hydroxyde adjuvant but does not show the outcome with regard to % seroprotection. From this concludes that the present set of claims (claims 1-15) can only partially claim this priority, i.e. only when SEQ ID NOs. are referred to that are disclosed in EP16161068. Finally, it is noted that EP16161068 does not relate to the subject-matter of present claims 16 and 17 (relating to '*PS at amounts too low to detect*'). Thus, claims 16 and 17 are not entitled to the priority of 18.03.2016.

The third and fourth priorities were both filed on 23-06-2016.

EP16176025 (PRIO3) is the first disclosure of the idea to use the successful JEV production system IXARIO® as basis for the production of Zika virus. This includes the use of protamine sulfate to remove contaminating DNA and surprisingly as well immature and non-infectious virus particles. The aspect of a purity with '*PS at amounts too low to detect*' is not explicitly mentioned. Therefore, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 23-06-2016. PRIO3 also mentions for the first time the Zika strain H/PF/2013 (SEQ ID NO: 13 of the present application, SEQ ID NO: 13 in PRIO3). In order to demonstrate or confirm complete entitlement of present claims 1-15 to claim this priority, the applicant will be asked to identify for each SEQ ID NO: in the claims, the corresponding sequence in EP16176025. If this is not possible, this priority can only be partially claimed.

EP16176049 (PRIO4) deals with the purification of viruses, the examples being directed at Flaviviridae, Zika virus being one of them. Example 2 addresses virus purity after PS treatment. However, it does not relate to the subject-matter of present claims 16 and 17 (relating to '*PS at amounts too low to detect*'). Thus, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 23-06-2016.

EP16182845 (PRIO5) of 04-08-2016 addresses the purity of the vaccine with regard to protamine salt and the introduction of an improved sucrose gradient. It will need to be discussed if the disclosures made in EP16182845 are sufficient to entitle the application to the priority date of 04-08-2016. At the moment, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 04-08-2016.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1	B. D. COX ET AL: "Predicting Zika virus structural biology: Challenges and opportunities for intervention", ANTIVIRAL CHEMISTRY & CHEMOTHERAPY., vol. 24, no. 3-4, 1 August 2015 (2015-08-01), pages 118-126, XP055328128
D2	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987
D3	US 8 765 148 B2 (WIZEL BENJAMIN ET AL) 1 July 2014 (2014-07-01) cited in the application

D1 is regarded as being the prior art closest to the subject-matter of claim 1, and discloses that Zika virus is in the center of an emerging crisis that requires the development of a vaccine. D1 does not relate to an inactivated Zika virus vaccine. Hence, claims 1 and dependent and related claims 2-17 are new.

The problem to be solved by the present invention may be regarded as the provision of a safe and efficient Zika virus vaccine.

The solution to this problem proposed in claim 1 of the present application, namely an inactivated Zika virus vaccine that is able to confer seroprotection on at least 70% of subjects is not considered as involving an inventive step because from D1 it was already known that Zika virus belongs to the genus Flavivirus, like DENV or JEV (D1: p. 119, left col. §2). From D2 it was further known, how to produce a successful Flavivirus vaccine. Hence it is considered that the skilled person looking to develop a

Zika virus vaccine would turn to D2 to find a strategy for the development of a Flavivirus vaccine with a reasonable expectation of success and without the exercise of inventive skill, in order to solve the problem posed.

Having regard to the dependent and related claims, it appears that none of the claims relates to features that are not readily derivable from either document D1 or D2 or document D3 (which discloses the specific adjuvants of claims 13 and 14). This includes at least some of the sequences listed in claims 3 and 4 (note that D1 already states that many Zika virus sequences are known). Finally, it is noted that the sequences of claims 3 and 4 are not shown in the application as having any surprising effect with regard to the development of a vaccine. Hence, they are obvious selections that become available to the skilled person as soon as a new strain is identified and published, and cannot establish an inventive step.

Re Item VI

Certain documents cited

D4	WO 2016/145149 A1 (THE US SECRETARY OF THE ARMY ON BEHALF OF THE WALTER REED ARMY INST OF) 15 September 2016 (2016-09-15)
D5	WO 2017/009873 A1 (BHARAT BIOTECH INT LTD) 19 January 2017 (2017-01-19)
D6	CN 105 749 268 A (SINOVAC RES & DEV CO LTD) 13 July 2016 (2016-07-13)
D7	RAFAEL A. LAROCCA ET AL: "Vaccine protection against Zika virus from Brazil", NATURE, vol. 536, no. 7617, 28 June 2016 (2016-06-28), pages 474-478, XP055314483

WO 2016/145149 (D4) published on 15 September 2016 claims the priority of 11-03-2015. Its priority is, thus before the first priority of the present application.

It discloses a vaccine comprising at least on inactivated Dengue virus and one inactivated non-Dengue virus, for example Zika virus. The Flavivirus is grown in Vero cells, treated with protamine sulfate (2mg/ml), purified over a sucrose gradient and activated by formaldehyde treatment (5mM formalin at 22°C for 10 days). The vaccine is formulated with aluminium hydroxyde as adjuvant.

WO 2017/009873 (D5) published on 19 January 2017 claims the priority of 16 July 2015. Its priority is, thus before the first priority of the present application.

It discloses a vaccine comprising an inactivated Zika virus, f. ex. formaldehyde deactivated.

CN 105 749 268 (D6) published on 13 July 2016 claims the priority of 11 April 2016. Its priority is, thus before the priorities PRIO3 and PRIO4 of the present application.

It discloses an inactivated (f. ex. formaldehyde inactivated) Zika virus vaccine. Purification is done by sucrose gradient centrifugation and the vaccine may be formulated with an aluminium salt.

Larocca et al. (D7) published on 28 June 2016, discloses an inactivated Zika virus vaccine (PIV) which shows complete protection after one single administration in a mouse model. The strain used was Puerto Rican PRVABC59 Zika virus which is SEQ ID NO: 4 in the present application, it was passaged in Vero cells, formalin inactivated (0.05%, 22°C for 7 days) and formulated with alum.

Re Item VIII

Certain observations on the international application

Claim 1 does not meet the requirements of Article 6 PCT because the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved ('*seroprotection on at least 70% of subjects*'), which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result.

It is clear from the description and the examples, that the advantageous immunogenicity and safety of the inactivated Zika virus vaccine lies in the preparation and purification and not in the strain selection or the corresponding genomes and E proteins. Hence, dependent claims 2-4 cannot provide features to overcome the clarity objection concerning claim 1.

Dependent claims 5-9 address the inactivation step. The respective features ('*inactivated by* ...'), however, relate to a method and cannot *per se* serve to characterize a product. Hence, also claims 5-9 are unclear in the sense of Article 6 PCT.

Dependent claims 10-15 relate to the adjuvant and/or excipient. Adjuvants and excipients often have an effect on the performance of a vaccine but they cannot serve to characterize the inactivated virus particle in order to overcome the objection concerning claim 1.

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2016/082664	23.12.2016	23.12.2015

International Patent Classification (IPC) or both national classification and IPC
INV. A61K39/12 C07K14/005

Applicant
VALNEVA AUSTRIA GMBH

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Lewis, Birgit Telephone No. +49 89 2399-0

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☐ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

   see separate sheet

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-17
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item II

Priority

The present application claims 5 priorities.

The first priority (23.12.2015) is not valid because patent application EP15202585 does not relate to Zika virus at all.

The second priority is dated 18.03.2016. EP16161068 relates to Zika virus including an inactivated Zika virus but not to a genome of Zika strain H/PF/2013 (SEQ ID NO: 13 of the present application, accession number KJ776791.1). Example 2 discloses virus growth and purification including a concentration step by chilling in ice for 2 hours after adding 0.5-2mg of protamine sulfate and ultracentrifugation on sucrose gradients at 38.000 g for 18 hours followed by filtration through a 0.22 µm filter. Example 3 relates to virus inactivation by formaldehyde (0.018% at 22°c or 4°C). Example 4 tests the immunogenicity of the virus with aluminium hydroxyde adjuvant but does not show the outcome with regard to % seroprotection. From this concludes that the present set of claims (claims 1-15) can only partially claim this priority, i.e. only when SEQ ID NOs. are referred to that are disclosed in EP16161068. Finally, it is noted that EP16161068 does not relate to the subject-matter of present claims 16 and 17 (relating to '*PS at amounts too low to detect*'). Thus, claims 16 and 17 are not entitled to the priority of 18.03.2016.

The third and fourth priorities were both filed on 23-06-2016.

EP16176025 (PRIO3) is the first disclosure of the idea to use the successful JEV production system IXARIO® as basis for the production of Zika virus. This includes the use of protamine sulfate to remove contaminating DNA and surprisingly as well immature and non-infectious virus particles. The aspect of a purity with '*PS at amounts too low to detect*' is not explicitly mentioned. Therefore, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 23-06-2016. PRIO3 also mentions for the first time the Zika strain H/PF/2013 (SEQ ID NO: 13 of the present application, SEQ ID NO: 13 in PRIO3). In order to demonstrate or confirm complete entitlement of present claims 1-15 to claim this priority, the applicant will be asked to identify for each SEQ ID NO: in the claims, the corresponding sequence in EP16176025. If this is not possible, this priority can only be partially claimed.

EP16176049 (PRIO4) deals with the purification of viruses, the examples being directed at Flaviviridae, Zika virus being one of them. Example 2 addresses virus purity after PS treatment. However, it does not relate to the subject-matter of present claims 16 and 17 (relating to '*PS at amounts too low to detect*'). Thus, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 23-06-2016.

EP16182845 (PRIO5) of 04-08-2016 addresses the purity of the vaccine with regard to protamine salt and the introduction of an improved sucrose gradient. It will need to be discussed if the disclosures made in EP16182845 are sufficient to entitle the application to the priority date of 04-08-2016. At the moment, this Authority is of the opinion that claims 16 and 17 are not entitled to the priority of 04-08-2016.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1	B. D. COX ET AL: "Predicting Zika virus structural biology: Challenges and opportunities for intervention", ANTIVIRAL CHEMISTRY & CHEMOTHERAPY., vol. 24, no. 3-4, 1 August 2015 (2015-08-01), pages 118-126, XP055328128
D2	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987
D3	US 8 765 148 B2 (WIZEL BENJAMIN ET AL) 1 July 2014 (2014-07-01) cited in the application

D1 is regarded as being the prior art closest to the subject-matter of claim 1, and discloses that Zika virus is in the center of an emerging crisis that requires the development of a vaccine. D1 does not relate to an inactivated Zika virus vaccine. Hence, claims 1 and dependent and related claims 2-17 are new.

The problem to be solved by the present invention may be regarded as the provision of a safe and efficient Zika virus vaccine.

The solution to this problem proposed in claim 1 of the present application, namely an inactivated Zika virus vaccine that is able to confer seroprotection on at least 70% of subjects is not considered as involving an inventive step because from D1 it was already known that Zika virus belongs to the genus Flavivirus, like DENV or JEV (D1: p. 119, left col. §2). From D2 it was further known, how to produce a successful Flavivirus vaccine. Hence it is considered that the skilled person looking to develop a

Zika virus vaccine would turn to D2 to find a strategy for the development of a Flavivirus vaccine with a reasonable expectation of success and without the exercise of inventive skill, in order to solve the problem posed.

Having regard to the dependent and related claims, it appears that none of the claims relates to features that are not readily derivable from either document D1 or D2 or document D3 (which discloses the specific adjuvants of claims 13 and 14). This includes at least some of the sequences listed in claims 3 and 4 (note that D1 already states that many Zika virus sequences are known). Finally, it is noted that the sequences of claims 3 and 4 are not shown in the application as having any surprising effect with regard to the development of a vaccine. Hence, they are obvious selections that become available to the skilled person as soon as a new strain is identified and published, and cannot establish an inventive step.

Re Item VI

Certain documents cited

D4	WO 2016/145149 A1 (THE US SECRETARY OF THE ARMY ON BEHALF OF THE WALTER REED ARMY INST OF) 15 September 2016 (2016-09-15)
D5	WO 2017/009873 A1 (BHARAT BIOTECH INT LTD) 19 January 2017 (2017-01-19)
D6	CN 105 749 268 A (SINOVAC RES & DEV CO LTD) 13 July 2016 (2016-07-13)
D7	RAFAEL A. LAROCCA ET AL: "Vaccine protection against Zika virus from Brazil", NATURE, vol. 536, no. 7617, 28 June 2016 (2016-06-28), pages 474-478, XP055314483

WO 2016/145149 (D4) published on 15 September 2016 claims the priority of 11-03-2015. Its priority is, thus before the first priority of the present application.

It discloses a vaccine comprising at least on inactivated Dengue virus and one inactivated non-Dengue virus, for example Zika virus. The Flavivirus is grown in Vero cells, treated with protamine sulfate (2mg/ml), purified over a sucrose gradient and activated by formaldehyde treatment (5mM formalin at 22°C for 10 days). The vaccine is formulated with aluminium hydroxyde as adjuvant.

WO 2017/009873 (D5) published on 19 January 2017 claims the priority of 16 July 2015. Its priority is, thus before the first priority of the present application.

It discloses a vaccine comprising an inactivated Zika virus, f. ex. formaldehyde deactivated.

CN 105 749 268 (D6) published on 13 July 2016 claims the priority of 11 April 2016. Its priority is, thus before the priorities PRIO3 and PRIO4 of the present application.

It discloses an inactivated (f. ex. formaldehyde inactivated) Zika virus vaccine. Purification is done by sucrose gradient centrifugation and the vaccine may be formulated with an aluminium salt.

Larocca et al. (D7) published on 28 June 2016, discloses an inactivated Zika virus vaccine (PIV) which shows complete protection after one single administration in a mouse model. The strain used was Puerto Rican PRVABC59 Zika virus which is SEQ ID NO: 4 in the present application, it was passaged in Vero cells, formalin inactivated (0.05%, 22°C for 7 days) and formulated with alum.

Re Item VIII

Certain observations on the international application

Claim 1 does not meet the requirements of Article 6 PCT because the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved ('*seroprotection on at least 70% of subjects*'), which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result.

It is clear from the description and the examples, that the advantageous immunogenicity and safety of the inactivated Zika virus vaccine lies in the preparation and purification and not in the strain selection or the corresponding genomes and E proteins. Hence, dependent claims 2-4 cannot provide features to overcome the clarity objection concerning claim 1.

Dependent claims 5-9 address the inactivation step. The respective features ('*inactivated by* ...'), however, relate to a method and cannot *per se* serve to characterize a product. Hence, also claims 5-9 are unclear in the sense of Article 6 PCT.

Dependent claims 10-15 relate to the adjuvant and/or excipient. Adjuvants and excipients often have an effect on the performance of a vaccine but they cannot serve to characterize the inactivated virus particle in order to overcome the objection concerning claim 1.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 7/00, 7/08, A61K 39/12 | A1

(11) International Publication Number: **WO 99/11762**

(43) International Publication Date: 11 March 1999 (11.03.99)

(21) International Application Number: PCT/KR98/00259

(22) International Filing Date: 25 August 1998 (25.08.98)

(30) Priority Data:

1997/42001	28 August 1997 (28.08.97)	KR
1997/42002	28 August 1997 (28.08.97)	KR

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(57) Abstract

The present invention relates to an attenuated Japanese encephalitis virus adapted to Vero cell by passages on Vero cell and a Japanese encephalitis vaccine comprising said attenuated virus.

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AN ATTENUATED JAPANESE ENCEPHALITIS VIRUS ADAPTED TO VERO CELL AND A JAPANESE ENCEPHALITIS VACCINE

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to an attenuated Japanese encephalitis virus adapted to Vero cell by passages on Vero cell and a Japanese encephalitis vaccine comprising said attenuated virus.

DESCRIPTION OF THE PRIOR ART

Japanese encephalitis (JE) is a mosquito-borne arboviral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually from that continent, but official reports undoubtedly underestimate the true number of cases (Okuno, T. *World Health Stat Q.* 3: 120-31,1978; Umenei, T. *et al. Bull World Health Org.* 63: 625-31, 1985). The illness may be manifested by a febrile headache syndrome, aseptic meningitis, or encephalitis and about half of the survivors tend to have permanent neurologic and psychiatric sequelae (Burke, D.S. *et al.* The Arbovirus: *Epidemiology and Ecology* 3:63-92, 1988; Monath, T.P. *Virology* 763-814, 1990).

JE virus is one of 66 Flaviviridae, enveloped, positive-sense, single stranded RNA viruses that largely are vector-borne (Chambers, T. J. *et al. Ann. Rev. Microbiol.* 44:649-88, 1990). Morphologically, flaviviruses are spherical, approximately 40 nm in diameter, are composed of a lipid bilayer surrounding a neucleocapsid containing 11-kb genome complexed with a capsid (C) protein (Rice, C. M. *et al. Science* 229:726-33, 1985). Surface projections on the membrane are composed of glycosylated envelope (E)

and membrane (M) proteins. A pre-M glycoprotein, present in intracellular nascent virions, is cleaved to the M protein, found in mature extracellular virions. Important physiological activities are associated with the 53-kd E protein, including hemagglutination, viral neutralization, virion assembly, membrane fusion, and viral binding to cellular receptors (Koshini, E. *et al. Virol.* 188:714-20, 1992).

There are three JE vaccines for humans (Tsai, T. *et al. Vaccines* 671-713, 1993). Of the three, only inactivated JE vaccine produced in mouse brain is available internationally. One manufacturer, the Research Foundation for Microbial Diseases of Osaka University (Biken) produces most of the inactivated JE vaccine distributed internationally; that vaccine was licensed in 1992 in the USA where it is distributed by Connaught Laboratories, Inc., as JE-VAX™. Inactivated and live attenuated JE vaccine prepared in primary hamster kidney (PHK) cells are distributed solely in China.

Inactivated JE vaccine produced in mouse brains was licensed in Japan in 1954. Because it is produced by cerebral injection of infant mice, it is laborious to manufacture and concerns about the possibility of vaccine-related neurological side effect were raised. Though successive refinements in the manufacturing process have increased its purity and potency (Oya, A. *Vaccination Theory and Practice* 69-82, 1975; Oya, A. *Acta Pediatr Jpn.* 30:175-84, 1988; Takaku, K. *Biken J.* 11:25-39, 1968), a moderate frequency of local and mild systemic reactions have been reported until recently (Hoke, C.H. *et al. New Engl J Med.* 319:608-14, 1988; Poland, J.D. *et al. J Infect Dis.* 161:878-82, 1990; Sanchez, J.L. *et al. Lancet* 335:972-73, 1990). Local tenderness, redness, and/or swelling at the injection site occur in 20 % of vaccines. Mild systemic symptoms, chiefly headache, low-grade fever, myalgias, malaise, and gastrointestinal symptoms, are reported by 10 to 30 % of vaccines. An apparently new pattern of adverse reactions including urticaria, angioedema, respiratory distress, erythema multiforme, erythema nosodum and severe neurological disorders have been reported since 1989, principally among travellers vaccinated in Australia, Europe, and North America (Anderson, M.M. *et al. Lancet.* 337:1044, 1991; Ruff, T.A. *et al. Lancet* 228:881-2, 1991). In addition, in 1992 and 1995 Ohtaki reported seven children with

acute disseminated encephalomyelitis (ADEM) with changes on magnetic resonance images (MRI) after JE vaccination (Ohtaki, E. *et al. Pediatr Neurol.* 8:137-9, 1992; Ohtaki, E. *et al. J Neurol Neurosurg Psychiatry* 59:316-7, 1995). Also of note is that vaccination with rabies vaccine containing animal brain tissue has caused severe neurological complications (Plotkin, S.A. *et al. Vaccines* 661-2, 1994). For these reasons, the WHO has designated JE vaccine development as a high priority.

More recently, inactivated and live attenuated JE vaccine of China have proven to be effective, eliciting high titers of virus-neutralizing antibody and conferring solid protection (Tsai, T. *et al. Vaccines* 671-713, 1993). However, PHK cells in which Chinese vaccine were prepared are not approved by the World Health Organization (WHO) for viral vaccine production or licensed for human use by the developed countries. The principal disadvantage in using primary hamster cells for the production of vaccines is the uncertainty with regard to the quality of vaccine. Even if specific pathogen free hamsters are used, animals can unexpectedly become infected, being problematic for vaccine production. Occasionally an infection of this type could be undetected for a long time. With these criticisms, further controlled studies of the safety of the vaccine are required to allow confidence regarding its widespread use. Another disadvantage of the vaccine production from primary cells is the low rate of harvest of the virus and high cost without allowing mass production.

In view of the above, there is a need for new JE vaccine which is produced in standard cell lines such as Vero or human diploid cells that have been accepted as human vaccine substrates, with good cost effectiveness. Vero cells are transformed but non-tumorigenic cells derived from monkey kidney. The Vero cell line is more advantageous than any other standard cell line in that Vero cells are more readily adaptable to large scale cell culture and as a transformed cell has an infinite life time.

It has now been found that JE virus can be grown in Vero cell culture. Considerable efforts had been made in the field of JE vaccine to produce vaccine in standard cells which permit effecting cell cultures at a large volume. Nevertheless, virus

characterization including genetic stability, yield and process necessary for vaccine commercialization through cultivation with Vero cells had never met the requirements of human vaccine. Owing to these facts and to the difficulties of transposing a knowledge acquired in other virus cultures to JE virus, the prior art had not achieved success in the development of JE virus vaccine which is genetically stable and has a high immunogenic character from continuous cell lines. Among all these researches, none had resulted up to the present time in a new vaccine production which satisfies the criteria mentioned in this background.

The present invention suggests a development and a propagation of JE virus in continuous cell line, Vero cells for vaccine production which overcome previous problems in JE virus produced in mouse brain or primary cell lines. The present invention also identifies methodology developed to cultivate the JE virus and a downstream process for vaccine production with cost-effectiveness.

In addition, the present invention identifies methodology improved upon the previously commercialized JE vaccines in the following ways.

1. Safety: The invented virus did not acquire the virulence through the Vero cell cultivation, reducing the hazards of production and affording an additional level of safety to recipients beyond that furnished by stringent control over the virus-inactivation process. This advantage has never been provided by the previously commercialized JE vaccines.

2. Increased supply in safer production substrate: The JE vaccine of the present invention is produced in the absence of bovine serum, making high yields and inexpensive and scalable production which are not achieved in the the previously commercialized JE vaccines.

3. Less reactogenicity: No gelatin stabilizer is incorporated into the JE vaccine of the present invention, reducing the risk of vaccine reactions like those seen

with the existing vaccine (Saskaguchi M. et al. Vaccines 68-69, 1998). In addition, undesirable bovine-derived components, incorporated in the existing JE vaccines are effectively eliminated. Conclusively, this safety point of the present invention has never been provided by the previous JE vaccine.

4. Increased potency: The success of scalable production with Vero cells and the absence of supplements for production, as well as the effective purification, permits the first use of potent adjuvants in formulating the JE vaccine. Although the use of the adjuvants in the vaccine formulation has been applied in other vaccine, transposing this knowledge to the JE vaccine has been in difficulty since none of the existing JE vaccines assure it's safe production.

In conclusion, none had resulted up to the present time in a new vaccine which satisfies the aforementioned advantages in the commercialization of JE vaccine.

Therefore, an object of the present invention is to provide a safe and effective JE vaccine produced in standard cell substrate to increase its acceptability in many countries. A further object of the invention is to provide an effective process for producing a highly purified stable vaccine and formulating a vaccine which has a high immunogenic character with a small antigen amount.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides an attenuated Japanese encephalitis virus adapted to Vero cell by passages on Vero cell.The attenuated Japanese encephalitis virus of the present invention, which is referred to as CJ50003 herein, was deposited at the permanent collection of the Korean Culture Center of Microorganisms, Seoul, Korea, on April 20, 1998 under the Budapest Treaty of the international recognition of the deposit of microorganisms for the purpose of patent procedure, and a subculture thereof can be obtained from the repository under the accession number KCCM-10125.

In another aspect, the present invention provides a Japanese encephalitis vaccine comprising an attenuated Japanese encephalitis virus adapted to Vero cell by passages on Vero cell.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying drawings as follows:

Figure 1 shows passages and adaptation of JE virus SA14-14-2(PDK) in Vero cells. Virus passage 1 was harvested at 5 days after inoculation of Vero cell monolayer with 0.1 moi of SA14-14-2(PDK) strain. The JE virus titer was measured by plaque assay performed on Vero cell monolayers. Subsequent serial passages were conducted to 30 passages with virus passage 1 virus as starting material by successive virus infection and titration as described in Example 1.

Figure 2 shows multiple time harvest of JE virus CJ50003 in a roller bottle every other day from 3 to 17 days post infection. Vero cell monolayer were infected at a moi of 0.01 plaque forming unit (pfu) per cell. Virus was allowed to adsorb for 2 hrs at 35°C, then cells were washed with PBS three times, fed with 100 ml of serum-free EMEM and incubated at 35°C. Every 48 hrs from 3 days to 17 days post inoculation, culture supernatants were replaced with fresh serum-free EMEM. Virus infectivity titrations of the harvests were performed by plaquing on Vero-cell monolayers.

Figure 3 shows an analysis of JE virus CJ50003 purified by sucrose gradient ultracentrifugation by SDS-PAGE and Western blotting. Sixty ml of concentrated culture supernatant was applied to a forty ml of 15-60% sucrose gradient and centrifuged in a 45 Ti rotor at 22,000 rpm, 18 hrs., 12°C. Two ml samples were collected from the bottom of the tube and subjected to 4-20 % gradient SDS-PAGE and the resolved proteins were transferred to Nitrocellulose membranes. Proteins were

visualized by staining with Coomassie brilliant blue (Panel A) or silver nitrate (Panel B), and antigens were visualized by reaction with monoclonal antibody reactive against JE viral E protein (Panel C). Lane 1, pre-stained protein standards (Novex Seeblue™) representing molecular weights of 250, 98, 64, 50, 36, 30, 16, and 6 kDa from the top; Lane 2-10, fraction No. 3-11 from the bottom after ultracentrifugation; E, envelope protein; C, capsid protein; M, membrane protein.

Figure 4 shows formaldehyde inactivation kinetics of purified JE virus CJ50003. Purified JE virus preparations were inactivated with 0.018 % formaldehyde at 4°C or 22°C. Samples taken at the indicated times were titrated for their residual infectious virus by direct plaquing on Vero cell monolayers. Additionally, amplification assay was done to detect low levels of virus as follows. Duplicate flasks containing Vero cell monolayers were inoculated with samples from virus-inactivation time points. After a 2 hr adsorption period at 35°C, cells were refed and incubated at 35°C. Cells were refed at 7 days and at 14 days post-infection. The culture media were harvested and plaqued to detect infectious virus. Inactivation time points from two separate experiments are shown: 4°C (filled rectangle), 22°C (filled circle). Thermal inactivation (no formaldehyde) controls (open rectangle for 4°C and open circle for 22°C) were done in parallel.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a JE virus strain which has desirable properties for the preparation of the JE vaccine. Said virus is an attenuated virus and can propagate in the continuous cell line, Vero which is admitted by WHO as cell substrate for human vaccine production. Thus it is expected that said virus can be used for the preparation of more safe inactivated and live JE vaccines than current vaccines.

The present invention provides a vaccine that satisfies the present need. A JE virus has been adapted to Vero cell by serial passages at no higher than 35°C. Continued passages in Vero cell resulted in increase in virus titer, over 10^7 pfu per ml of culture

supernatant and reduced culture time to show peak virus titer. The invention concerns the development of a multiple time harvesting process with no serum requirement as supplement resulted in high yield of virus productivity, which is commercially feasible properties for the large-scale production of said vaccine with cost-effectiveness. According to the invention, the multiple harvesting process in virus cultivation is responsible for the reduced degree of cytopathic effect (CPE) of infected cells. The JE vaccine of the present invention contains an extremely small quantity of residual cell derived components owing to the reduced level of CPE. In addition, JE vaccine of the present invention is expected to afford enhanced immunogenicity and greater protection against disease than current JE vaccines. The purified JE vaccine of the present invention has a major advantage over current vaccines in that the purified viruses from the cultured Vero cells meet the development requirements for human vaccine.

The present invention also relates to the methods for the preparation of said vaccine. The methods can provide high productivity, purity and potency of said vaccine. The JE virus CJ50003 is obtained by subjecting JE virus SA14-14-2(PDK) to 4 passages or more of adapting in the Vero tissue culture cells at temperatures no higher than 35°C and selecting the cultured virus while monitoring the virus propagation based on the number of foci which were formed in Vero and/or LLC-MK2 cells. The virus obtained from that adaptation has a peak titer of at least $1x10^7$ pfu/ml of culture supernatant in Vero cell culture and reduced incubation period for harvest. The JE virus SA14-14-2 is an attenuated strain which is obtained by adapting a wild type JE virus SA14 from mosquito in the Primary Hamster Kidney (PHK) tissue culture cell and the Primary Dog Kidney (PDK) tissue culture cell (Kenneth H. Eckels, et al. *Vaccine* 6 513-518, 1988). But the PHK and PDK cells are not admitted by WHO, so they are not suitable for preparation of vaccines applicable to humans. The Vero cell is admitted by WHO for human use, so the Vero adapted JE virus strain, CJ50003, is a good basis for production of vaccine for humans.

It is known that SA14-14-2(PDK) virus belongs to flaviviridae and has the following physicochemical properties: single-stranded, positive-sense RNA genome

with 5' methylated end and 3' end with no poly A structure. The size of RNA genome is approximately 11 kb and the genome is in a combined state with nucleocapsid (C) protein of 13,500 Da. The virus is additionally comprised of membrane (M) protein of 8,700 Da, envelope (E) protein of 53,000 Da and non-structural proteins NS1, 2a, 2b, 3, 4a, 5 and the like.

The Vero adapted JE virus strain, CJ50003 was passed in Vero cell over 30 passages. The virus titer and the morphology of plaque were not varied through passaging, suggesting that the virus has stable phenotypic character.

To get an insight into the molecular basis for the biological characteristics of JE virus CJ50003, the physicochemical properties of the virus were analyzed. The sequence of the bases of the viral genome was determined by cDNA cloning and sequencing. As a result, it was discovered that three adenine bases of the 1032, 1506, and 1704 positions, and a guanine base of the 1769 position of E protein gene of JE SA14-14-2(PDK) virus were replaced by three guanines and a thymine in JE CJ50003 virus, respectively. Accordingly, the amino acid sequence of E protein was changed from threonine of the 19 position, threonine of the 177 position, lysine of the 243 position and glutamine of the 264 position to alanine, alanine, glutamate and histidine, respectively. The amino acid changes on the E protein were maintained through passaging the virus in Vero cell as long as our investigation lasted.

The JE virus CJ50003 did not kill mice when the viruses which have different number of passages in Vero cells, were injected to young mice intracerebrally. Accordingly, it can be said that the Vero adapted JE virus CJ50003 is an attenuated and stable virus strain which has no or little neurovirulence. It is one of the critical points to use said virus for the live JE virus vaccine and/or inactivated vaccine.

The present invention also provides a method for purifying virus from cell culture without freezing the crude or interim purity materials. Said method comprises the steps of removing cell debris, concentrating the virus, purifying the virus by

precipitation of the materials of cell origin and sucrose gradient ultracentrifugation, fractionating the gradients and assaying the fractions for virus. More specifically, the present invention provides a method for the production of purified JE virus, by propagating virus to high titer in continuous cell lines, in the presence or absence of serum protein supplements, purifying the virus by ultracentrifugation, and pooling the virus-positive fractions.

The said virus is propagated in Vero tissue culture cells. The confluently grown Vero cells in roller bottles are infected and incubated with the CJ50003 virus. Harvesting the virus can be done by the multiple harvesting method. The harvest of culture supernatant was started at the point of 2 or 3 days post infection according to moi of infection, and the fresh medium was refed to the culture. After 2 day incubation of the refed culture, the culture supernatant was harvested again. Harvesting can be repeated up to 4 times by 8 or 9 days post infection with the virus titer maintained over 10^7 pfu/ml of culture supernatant. The multiple harvesting method gave a high yield of virus per unit roller bottle, so it makes this invention more compatible with the laws of market. Furthermore, the process is responsible for the reduced degree of CPE of infected cells. The reduced level of CPE contributes to extremely low level of residual cell derived components in JE vaccine of the present invention. The harvested culture supernatants can be stored at 4°C until the purification started. The clarification of the harvested culture supernatants can be accomplished by common methods known in the art including low-speed centrifugation, for example, at 1500 g for 10 min, and/or by filtration through a filter of pore size of 0.45. The harvested culture fluid is stored at 4°C until concentration. For the concentration of the virus, the culture fluid is ultrafiltrated and the retentate is collected. In another method for concentration, the polyethylene glycol (PEG) 8000 is dissolved in the culture fluid up to 10% and the precipitate is dissolved in a proper buffer, for example phosphate-buffered saline (PBS, pH 7.0). The protamine sulfate precipitation is performed for removing DNA or other materials which originated from the cell, which can be accomplished by addition of protamine sulfate to concentrated virus solution and high speed centrifugation, for example, at 12,000 g, for 5 min. For further purification of the virus, density gradient

ultracentrifugation is performed on 15-60% continuous or multi-step sucrose gradients. The sucrose gradient is fractionated and the fractions are assayed for the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as immunoassays. The fractions for further processing are pooled on the basis of high virus titer and low level of other impurities. The purity of the pooled purified virus was estimated by testing for Vero cell originated chromosomal DNA and protein. The results showed that contents of host cellular DNA and protein are as low as 2.5 pg and 2 ng per 5 μg of purified JE virus, respectively, which demonstrated the purification methods described above effectively removed other impurities from viral antigen. JE virus yield from 1 L of infected culture fluid is estimated to be about 2.3 milligrams.

The present invention also provides a method of inactivating JE virus to destroy its infectivity while preserving its antigenicity. Said method comprises adding an effective quantity of formaldehyde and incubating said virus with said agent in certain conditions such that said virus is inactivated. Specifically, the fraction pool was diluted to appropriate protein concentration with a proper buffer such as PBS and the formaldehyde was added to the diluted fraction pool. The incubation with formaldehyde was performed at 22°C or 4°C. At least 4 or 46 days were required to fully destroy viral infectivity without loss of viral antigenicity at 22°C or 4°C, respectively. The inactivation process of JE virus at 22°C was preferably chosen for simplicity in large scale culture and incubation time was extended to 7 days for a safety margin. However, examples of inactivating agents which were effective include but are not limited to formaldehyde. In general, this can be achieved by chemical or physical means. Chemical inactivation can be effected by treating the viruses, for example, with enzymes, β-propionlactone, ethylene-imine or a derivative thereof, and an organic solvent such as Tween, Triton, sodium deoxycholate, and sulphohetain. If necessary, the inactivating substance is neutralized afterwards; material inactivated with formaldehyde can, for example, be neutralized with thiosulphate. Physical inactivation can preferably be carried out by subjecting the viruses to energy-rich radiation, such as UV light, X-radiation or gamma-radiation.

The JE vaccines are prepared as injectables, either as liquid solution or suspension. It is possible to add a stabilizing agent such as carbohydrates (sorbitol, mannitol, starch, sucrose, dextran, glucose, etc), proteins (albumins, casein, etc), an agent containing proteins (bovine serum, skim milk, etc) and buffers (such as alkali metal phosphate). The preparation can be lyophilized after adding a stabilizer and it can be vacuum or nitrogen stored. If desired, one or more compounds with an adjuvant action can be added. Suitable compounds for this purpose are, for example, aluminum hydroxide, phosphate or oxide, mineral oil (such as Bayol, Marcol 52) and saponins. In addition, if desired, one or more emulsifiers, such as Tween and span, is also added to the virus materials.

The effectiveness of an adjuvant was determined by measuring the amount of neutralizing antibodies directed against the virus resulting from administration of the inactive virus in vaccines which are also absorbed to an adjuvant. Examples of adjuvant which was effective include but is not limited to alum hydroxide. The obtained vaccine was investigated for efficacy by the plaque reduction neutralization test (PRNT) with the sera of said vaccine immunized mice and direct challenge of immunized mice with a neurovirulent virus. As a result, it was shown that the said vaccine had the same as or better efficacy of eliciting neutralizing antibody than comparable vaccines.

To investigate possible changes in immunogenicity of Vero adapted viruses with different passage numbers, the vaccines were prepared in different passage numbers and the efficacy of each vaccine was compared. There was no remarkable difference in efficacy among the vaccines prepared from viruses with different passage numbers in spite of successive passing in Vero cell. Thus it can be said that the Vero adapted JE virus strain, CJ50003, has stable immunogenicity.

The following examples illustrate the attenuated JE virus adapted to Vero cell according to the present invention and the JE vaccine comprising said attenuated virus according to the present invention. From the foregoing description and the following examples, it is believed that those skilled in the art would be able to carry out the

invention to the fullest extent.

EXAMPLE 1

Adaptation of SA14-14-2(PDK) virus in Vero cell

JE SA14-14-2 (PDK), SA14-14-2 virus in dog kidney cell culture passage 8 was used to initiate serial passages in Vero cell culture. The Vero cell monolayers were inoculated with JE SA14-14-2 (PDK) at an moi of 0.1 pfu per cell. The infected cell cultures were grown in 25 cm^2 culture flasks containing 5 ml of nutrient media consisting of Eagle's minimal essential media supplemented with 10 % fetal bovine serum in an atmosphere of about 5 % CO_2 in air and at a temperature no higher than about 35°C, typically at from about 32°C to about 35°C, with about 35°C being preferred. Viral growth was monitored by microscopic observation of cytopathic effect (CPE) and various assay for the presence of viral antigen including hemadsorption assay (HA), plaque assay, and enzyme linked immunoadsorbant assay (ELISA). JE virus was harvested at day 5 post infection when the culture showed peak of virus titer, clarified by centrifugation. The single plaque was purified from the clarified supernatant and amplified in Vero cells. The amplified virus was re-infected to Vero cell for further passages. Subsequent serial passages were conducted up to 30 passages by successive virus infection, titration, and plaque-purification as described above. As shown in Figure 1, the virus titers reached about $4X10^7$ pfu per ml of culture supernatant with 4 passages in Vero cells and maintained close to this level in further passages. Besides, the optimal period for viral harvest was reduced from 5 days at passage 1 to 2 - 3 days at passage 4. A significant increase in virus yield, about 10^5 pfu/ml to over 10^7 pfu/ml and a decrease in incubation time resulted in the selection of the JE passage 4 in Vero cells as starting material of choice for the preparation of a candidate JE vaccine. The JE passage 4 in Vero cells was labelled as CJ50003 (Vero, PS4). Abbreviation PS means virus passage number in designated cell.

EXAMPLE 2

Characterization of CJ50003 virus; Sequencing of the envelope gene and

neurovirulence study

As an effort to give an insight into the molecular basis for the biological characteristic of CJ50003 strain, the 1500 nucleotide sequence encoding the envelope(E) gene which possesses major neutralizing epitopes were determined and compared with those of the parent vaccine strains, SA14-14-2(PDK), SA14-14-2(PHK) and an wild type SA14 virus (Aihira, S. *et al. Virus Genes*, 5:95-109, 1991; Ni, H. *et al . J Gen Virol.* 76:401-407, 1995; Ni, H. *et al. J Gen Virol.* 76:409-413, 1995; Nitayaphan, S. *et al. Virology* 177:541-542, 1990). CJ50003 virus (Vero, PS4) was used for sequence analysis. This revealed that the C-terminal region (amino acid 280-500) shows complete conservation, while the N-terminal region (amino acid 1-279) shows sequence variation among the virus strains. Mutations in the N-terminal region are almost evenly distributed. Nucleotide sequence of the E protein gene of CJ50003 differed from SA14/CDC by 8 nucleotides and 7 amino acids whereas SA14-14-2(PDK) differed from SA14/CDC by 7 nucleotides and 5 amino acids. The results were summarized in Table 1.

The sequence of CJ50003 virus differed from the published sequence of SA14-14-2(PDK) virus at 5 positions: nucleotide changes at positions 1032, 1506, 1704 and 1769 resulted in 4 amino acid differences between SA14-14-2(PDK) and CJ50003 viruses: nucleotide changes at position 989 did not result in amino acid substitution. Higher passages of CJ50003 virus, i.e. passage 15 and 30 revealed no additional nucleotide changes. There were, therefore 5 distinct nucleotide and 4 amino acid changes between CJ50003 and parent virus, SA14-14-2(PDK) and these changes were stable on passage of this virus in cell culture. The Lys residue at 243 in the SA14-14-2(PDK), which is uniquely different compared with other attenuated JE viruses were substituted with Glu residue in CJ50003.

CJ50003 sequence also differed from the published sequence of SA14-14-2(PHK) virus (Aihira, S. *et al. Virus Genes*, 5:95-109, 1991). The nucleotide difference at 1032 caused amino acid difference at position E19 but the change at

nucleotide position 989 did not result in amino acid substitution. Nucleotides at 1506 and 1704 in CJ50003 virus were the same as those present in the SA14-14-2(PHK) at these positions while different from the SA14-14-2(PDK) at those positions. The pattern of substitutions through the N-terminal region of the CJ50003 and SA14-14-2(PHK) E gene is almost same except for amino acid substitution at E19.

SA14-14-2(PHK), SA14-14-2(PDK) and CJ50003 viruses have 4 identical amino acid substitutions compared with the sequence of the parent SA14 virus at position E107, E138, E176 and E279. Of those the amino acids at position E138 and E176 (Ni, H. *et al. J Gen Virol.* 76:409-413, 1995), which were known to contribute to attenuation were still conserved after Vero adaptation, suggesting that CJ50003 did not lose it's attenuated character.

Table 1. Comparison of nucleotide and amino acid sequences among JE virus strains, SA14, SA14-14-2(PHK), SA14-14-2(PDK), and CJ50003.

Position		Nucleotide						Amino Acid					
NT	AA	SA14/ USA	SA14/ CDC	SA14/J AP	SA14-14-2/PHK	SA14-14-2/PDK	CJ50003	SA14/ USA	SA14/ CDC	SA14/J AP	SA14-14-2/PHK	SA14-14-2/PDK	CJ50003
989	E4	G	G	G	U	U	G	Leu	Leu	Leu	Leu	Leu	Leu
1032	E19	A	A	A	A	A	G	Thr	Thr	Thr	Thr	Thr	Ala
1052	E25	G	A	A	A	A	A	Leu	Leu	Leu	Leu	Leu	Leu
1061	E28	U	U	U	C	C	C	Asp	Asp	Asp	Asp	Asp	Asp
1217	E80	C	C	U	U	C	C	Ala	Ala	Ala	Ala	Ala	Ala
1296	E107	C	C	C	U	U	U	Leu	Leu	Leu	Phe	Phe	Phe
1389	E138	G	G	G	A	A	A	Glu	Glu	Glu	Lys	Lys	Lys
1503	E176	A	A	A	G	G	G	Ile	Ile	Ile	Val	Val	Val
1506	E177	A	A	A	G	A	G	Thr	Thr	Thr	Ala	Thr	Ala
1704	E243	G	G	G	G	A	G	Glu	Glu	Glu	Glu	Lys	Glu
1708	E244	G	A	A	A	A	A	Glu	Gly	Gly	Gly	Gly	Gly
1769	E264	G	G	G	A	G	U	Gln	Gln	Gln	His	Gln	His
1813	E279	A	A	A	U	U	U	Lys	Lys	Lys	Met	Met	Met
1921	E315	C	U	C	U	U	U	Ala	Val	Ala	Val	Val	Val
1977	E334	C	C	U	C	C	C	Pro	Pro	Ser	Pro	Pro	Pro
2293	E439	A	G	A	G	G	G	Lys	Arg	Arg	Arg	Arg	Arg
2441	E488	G	A	G	A	A	A	Gly	Gly	Gly	Gly	Gly	Gly

AA: amino acid; NT: nucleotide position strains.

CJ50003 and the parent SA14-14-2(PDK) were tested for their mice neurovirulence by intracerebral (i.c.) injection into the 4-week-old BALB/c mice. The results are shown in Table 2. The lethality for young adult mice is not significantly different between SA14-14-2(PDK) and CJ50003 viruses which is very low compared to that of wild type SA14 virus. Thus it seems to be that the introduction to the Vero cell substrate did not provide a neurovirulent phenotype to the SA14-14-2(PDK) and CJ50003 virus still has attenuated character.

Table 2. Intracerebral virulence of 4-week-old mice inoculated with Vero-passaged CJ50003 viruses. PS represents passage in PDK or Vero cell.

Virus	PFU Inoculum	log LD_{50} ml^{-1}	LD_{50}/PFU ratio
SA14(PDK, PS3)	2 x 10(7)	6.5	0.17[a]
SA14-14-2(PDK,PS8)	1.3 x 10(6)	<1.5[b]	<0.00002[a]
CJ50003(Vero, PS6)	3.4 x 10(7)	<1.5[b]	<0.000001
CJ50003(Vero, PS15)	3.2 x 10(7)	<1.5[b]	<0.000001
CJ50003(Vero, PS30)	3.6 x 10(7)	<1.5[b]	<0.000001

a: Kenneth H. Eckels *et al* (*Vaccine* 6:513-518, 1988).

b: 0/10 mice died after inoculation with undiluted virus.

The volume of inoculum for i.c. injection is 0.03 ml per mouse.

EXAMPLE 3

Virus growth and purification

The production seed was prepared in virus passage 5 in Vero cell [CJ50003 (Vero, PS5)]and stored in deep-freezer. Vero cells were grown in Eagle's minimal essential medium (EMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco). Roller bottle cultures of Vero cell monolayers were infected with production seed virus at an moi of 0.01 to 0.1 pfu per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM not containing serum and incubated at 35°C. In infected Vero cell cultures, virus reached titers of around 10^7 to 10^8 pfu/ml at 2 or 3 days post infection. While virus harvests were taken 4 times at 2 day intervals

until 8 or 9 days post infection starting from 2 or 3 days post infection, virus titers were still maintained over 10^7 pfu/ml with very weak CPE. But after 9 days post infection, the titers were under 10^7 pfu/ml (Figure 2). The pooled harvests were centrifuged at 8,000 rpm for 15 minutes and supernatants were filtered through a 0.45 μm filter. Virus culture supernatants were concentrated by ultrafiltration (Ultrasette, Filtron, 100k) or precipitation with PEG. The virus precipitated by PEG was collected by centrifugation and suspended in PBS or STE (10 mM Tris pH 7.2, 1 mM EDTA, 150 mM NaCl) buffer. Retentate after ultrafiltration was concentrated to 250 ml and the cassette was washed with 100 ml of PBS. Virus concentrates were chilled in the ice for 2 hours after adding 0.5 - 2 mg/ml of protamine sulfate and the supernatants obtained by centrifuging at 10,000 rpm for 5 minutes. The concentrated viruses were purified by ultra-centrifugation on sucrose gradients. The ultracentrifugation was carried out at 38,000 g for 18 hours. Fractions were subjected to electrophoresis on polyacrylamide gels containing the detergent sodium dodecyl sulfate (SDS-PAGE). The nucleocapsid protein (C, 13,500 Da), membrane protein (M, 8,700 Da) and envelope protein (E, 53,000 Da) bands were seen in the SDS-PAGE (Figure 3, panel A). Envelope antigens (E) were detected by Western blotting with mouse anti-JE virus monoclonal antibody (Figure 3, panel C). Virus positive fractions, fraction Nos.4 to 9, in which other protein bands except viral proteins were not apparent in silver stained gel (Figure 3, panel B) were pooled, and assayed for protein concentration by Lowry method. Detailed results are shown from two purifications from infected Vero cultures either concentrated with tangential flow ultrafiltration or by PEG8000 precipitation (Tables 3 and 4). Purified virus was diluted with two volumes of PBS, added to the final 0.01% of Tween80, and filtered through a 0.22 μm filter.

Table 3. Purification of JE virus by concentration with tangential flow ultrafiltration.

Sample	Total Volume (ml)	Total pfu	%Yield (pfu)	Total protein (mg)	%Yield (protein)	Specific Activity (pfu/mg)
Pooled culture supernatant	10,000	4.4×10^{11}	100	600	100	7.3×10^7

Filtron concentrate	200	$4.0x10^{11}$	90	280	47	$1.4x10^{8}$
Sucrose gradient pool	500	$3.8x10^{11}$	86	42	7	$9.0x10^{9}$
0.22 μ filtration	50	$2.4x10^{11}$	55	23	3.8	$1.0x10^{10}$

Table 4. Purification of JE virus by concentration with PEG8000 precipitation.

Sample	Total Volume (ml)	Total pfu	%Yield (pfu)	Total protein (mg)	%Yield (protein)	Specific Activity (pfu/mg)
Pooled culture supernatant	10,000	$4.4x10^{11}$	100	600	100	$7.3x10^{7}$
PEC precipitate	200	$2.7x10^{11}$	61	40	6.7	$6.8x10^{9}$
Sucrose gradient pool	500	$2.5x10^{11}$	56	15	2.5	$1.7x10^{10}$
0.22 μ filtration	50	$1.6x10^{11}$	41	12	2	$1.3x10^{10}$

The virus preparations were compared for relative purity using specific activity measurements (i.e., pfu/mg protein). Virus purified from concentrate by ultrafiltration had about the same activity as virus purified from concentrate by PEG8000 precipitation. Also the purity of the pooled purified viruses was estimated by testing for Vero cell originated chromosomal DNA and protein. The results showed that contents of host cellular DNA and protein are as low as 2.5 pg and 2 ng per 5 μg of purified JE virus respectively regardless of method of concentration, which demonstrated that both purification methods described above effectively removed other impurities from viral antigen. However in terms of protein yield of purified virus, the purification method using ultrafiltration is 2-fold better than the purification method involving PEG8000 precipitation.

EXAMPLE 4

Virus inactivation

Purified virus was either directly used for preparation of live attenuated vaccine after dialysis with PBS or inactivated with formaldehyde for preparation of inactivated vaccine. Inactivation with 0.018% formaldehyde was carried out at 22°C or 4°C. Samples were taken at regular intervals and assayed directly for infectious virus by plaque titration (Figure 4). Samples which were negative by direct plaque assay were subjected to blind passage on Vero cell monolayers in order to amplify low levels of virus and then re-plaqued. It was found that 4 days at 22°C or 46 days at 4°C were required for complete inactivation of infectivity (Tables 5 and 6). The antigenicity of the virus was monitored during inactivation by testing samples with the antigen spot blot assay and polyclonal antisera. By this assay there appeared to be no detectable losses in antigenicity after exposure to 0.018% formaldehyde for up to 10 days at 22°C or 15 days at 4°C.

Inactivation with formaldehyde under these conditions was carried out for at least 7 days at 22°C or 60 days at 4°C, giving a margin of safety. After inactivation, free formaldehyde in the samples was neutralized by the addition of 0.038% of sodium metabisulfite. Dialysis was carried out concurrently with PBS and then filtered through a 0.22 μm filter.

Table 5. Formaldehyde inactivation of JE virus, CJ50003 at 4°C

Day	JE virus (-HCHO)	JE virus (+HCHO)	Amplification
0	3.2×10^8	3.2×10^8	+
0.5	2.6×10^8	3.2×10^6	+
1	1.52×10^8	7.9×10^5	+
1.5	2.4×10^8	4.8×10^5	+
2	2.8×10^8	6.4×10^4	+
3	2.6×10^8	4.3×10^3	+
4	1.9×10^8	1300	+
5	2.4×10^8	285	+
6	2.8×10^8	480	+

7	1.5×10^8	200	+
11	1.5×10^8	0	+
22	1.4×10^8	0	+
32	1.2×10^8	0	+
46	1.0×10^8	0	-
60	1.0×10^8	0	-

Table 6. Formaldehyde inactivation of JE virus at 22°C

Hour	JE virus (-HCHO)	JE virus (+HCHO)	Amplification
0	3.2×10^8	3.2×10^8	+
3	3.0×10^8	1.3×10^6	+
6	2.7×10^8	1.1×10^5	+
12	2.5×10^8	3.5×10^5	+
24	1.2×10^8	140	+
36	1.2×10^8	0	+
48	1.2×10^8	0	+
72	1.1×10^8	0	+
96	1.1×10^8	0	-
360	1.0×10^8	0	-

EXAMPLE 5

Immunogenicity of CJ50003 purified, inactivated virus (PIV) and live attenuated virus (LAV) in mice

The immunogenicities of LAV and PIV were then tested in mice with previously commercialized Biken inactivated vaccine. Groups of 20 six-week-old BALB/c mice were immunized intraperitoneally (i.p.) with three kinds of immunogen. Immunization was done with two inoculations without an adjuvant at intervals of 2 weeks. Two weeks post second immunization sera were obtained from each group of mice, pooled and subsequently tested for the presence of neutralizing antibodies by PRNT method (Table

7). As shown in Table 7, there was no significant difference in neutralizing antibody titer between groups which received three kinds of immunogen.

Table 7. Induction of neutralizing antibodies in mice immunized with PIV or LAV

Immunogen	Dose	Titers of neutralizing antibodies [a]
PIV	5 μg	1:320
PIV	10 μg	1:320
LAV	5 μg	1:320
LAV	10 μg	1: 640
Biken vaccine [b]	1 dose	1: 320

a: Titer of neutralizing antibody is defined as the reciprocal of serum dilution resulting in 50% reduction of mouse brain passaged Nakayama virus plaques .

b: Biken vaccine 1 dose contains 5 μg of viral protein (TCA-precipitable) according to the manufacturer.

The Immunogenicity of PIV was further tested in mice. Adult, inbred mice were immunized with various dilutions of inactivated virus with or without an alum adjuvant. Groups of 20 six-week-old BALB/c mice were immunized subcutaneously with 500, 50, and 5 ng of PIV either in saline or saline with aluminum hydroxide (Rehydragel). Mice received two inoculations spaced 3 weeks apart. Sera were pooled from each group of mice at 3 weeks post second immunization, and tested for the presence of neutralizing antibodies with mouse brain passaged Nakayana strain as neutralized virus (Table 8). PIV was better than Biken vaccine in all doses and adjuvant significantly improved the immune response of mice to 50 and 500 ng of PIV about 4 and 8 fold, respectively.

Table 8. Comparison of the titer of neutralizing antibody in mice immunized with PIV with or without alum hydroxide.

Immunogen	Dose	Titers of neutralizing antibodies [a]

PIV	500 ng	1:160
PIV	50 ng	1:40
PIV	5 ng	1:20
PIV + alum	500 ng	1: 1280
PIV + alum	50 ng	1: 160
PIV + alum	5 ng	1: 20
Biken vaccine	1/10 dose	1: 80
Biken vaccine	1/100 dose	1: 10
Biken vaccine	1/1000 dose	1: 10

a: Titer of neutralizing antibody is defined as the reciprocal of serum dilution resulting in 50% reduction of mouse brain passaged Nakayama virus plaques.

b: Biken vaccine 1 dose contains 5 μg of viral protein (TCA-precipitable) according to the manufacturer.

The *in vivo* protective efficacy of PIV was then tested in BALB/c mice. For protection assays, groups of 10 three-week-old BALB/c mice were inoculated subcutaneously in the hindquarters with inactivated JE viruses in saline or saline with aluminum hydroxide (Rehydragel). Age-matched controls were inoculated with PBS or non-specific antigens in alum. Mice were boosted with an equivalent dose three weeks later. The mice were challenged at 3 weeks post immunization by intracranial inoculation with 500 pfu of the mouse neurovirulent JE virus (Nakayama, mouse brain adapted) contained in 30 μl of PBS. Challenged mice were monitored daily for morbidity and mortality for up to twenty-one days. As shown in Table 9, mice immunized with 50 ng of PIV showed 90% of protection. Furthermore, mice immunized with 50 and 5 ng of PIV mixed with alum showed 100% and 70% protection, respectively while 1/100 dose of Biken vaccine protected just 50% of immunized mice. In comparison, all mice in the control group became sick and died beginning at five to seven days post-challenge.

Table 9. Protection of vaccinated mice against challenge with Nakayama virus[a] Immunogen

Immunogen	Dose	Survivors
Control [b]	N/A	0/10
PIV	500 ng	10/10
PIV	50 ng	9/10
PIV	5 ng	3/10
PIV + alum	500 ng	10/10
PIV + alum	50 ng	10/10
PIV + alum	5 ng	7/10
Biken vaccine [c]	1/10 dose	10/10
Biken vaccine	1/100 dose	5/10
Biken vaccine	1/1000 dose	3/10

a: Mice immunized with 2 inoculations of test vaccines spaced 3 weeks apart, then challenged with 500 pfu of mouse-neurovirulent Nakayama virus.

b: Age-matched controls were inoculated with PBS or non-specific antigens in alum

c: Biken vaccine 1 dose contains 5 μg of viral protein (TCA-precipitable) according to the manufacturer .

To investigate immunologic stability of CJ50003 virus over Vero cell passages, viruses with various passage numbers in Vero cell were independently purified and the immunogenicities were evaluated by the method as described in Table 8. As shown in Table 10, there was no remarkable difference in the ability to elicit neutralizing antibodies among vaccines prepared from the viruses with different virus passage numbers in Vero cell, indicating that CJ50003 virus is very stable over Vero cell passages in terms of immunogenicity.

Table 10. Vaccine potencies prepared with JE viruses with different virus passage numbers in Vero cells

Immunogen [a]	Dose	Titers of neutralizing antibodies[b]	S.d.[c]
PIV - 4ps	0.5 μg	1:150	20
PIV - 6ps	0.5 μg	1:145	15
PIV - 15ps	0.5 μg	1:130	28
PIV - 20ps	0.5 μg	1:140	18
PIV - 30ps	0.5 μg	1:160	13

a: Immunogen (PIV -Xps); purified inactivated vaccine prepared with CJ50003 virus of which passage number in Vero cells is X.

b: Titer of neutralizing antibody is defined as the reciprocal of serum dilution resulting in 50% reduction of mouse brain passaged Nakayama virus plaques and mean values of results from three separate experiments ere taken. 50% endpoint is determined by Reed and Muench method.

c: Standard deviation

The results presented here suggest that both an inactivated JE virus vaccine and live attenuated vaccine using CJ50003 strain show promise. Relatively fast and efficient processes were developed for growing JE virus in Vero cell, concentrating and purifying them to a degree which may be suitable for human use and inactivating them without measurable loss in antigenicity. These preparations were found to be immunogenic and protective in mice.

WHAT IS CLAIMED IS:

1. An attenuated Japanese encephalitis virus adapted to Vero cell by passages on Vero cell.

2. The attenuated Japanese encephalitis virus according to claim 1 characterized in that multiplicity is more than 1x10(7) PFU/ml in Vero cells and LD_{50}/pfu for young adult mouse is less than 0.000001.

3. The attenuated Japanese encephalitis virus according to claims 1 or 2 wherein is CJ50003.

4. A Japanese encephalitis vaccine comprising the attenuated Japanese encephalitis virus according to claim 1.

5. The vaccine according to claim 4 wherein the virus is inactivated by an inactivating agent.

6. The vaccine according to claim 4 wherein the virus is live-attenuated JE virus untreated by an inactivating agent.

7. The vaccine according to claims 4, 5 or 6 which further comprises pharmaceutically acceptable additives.

Fig 1.

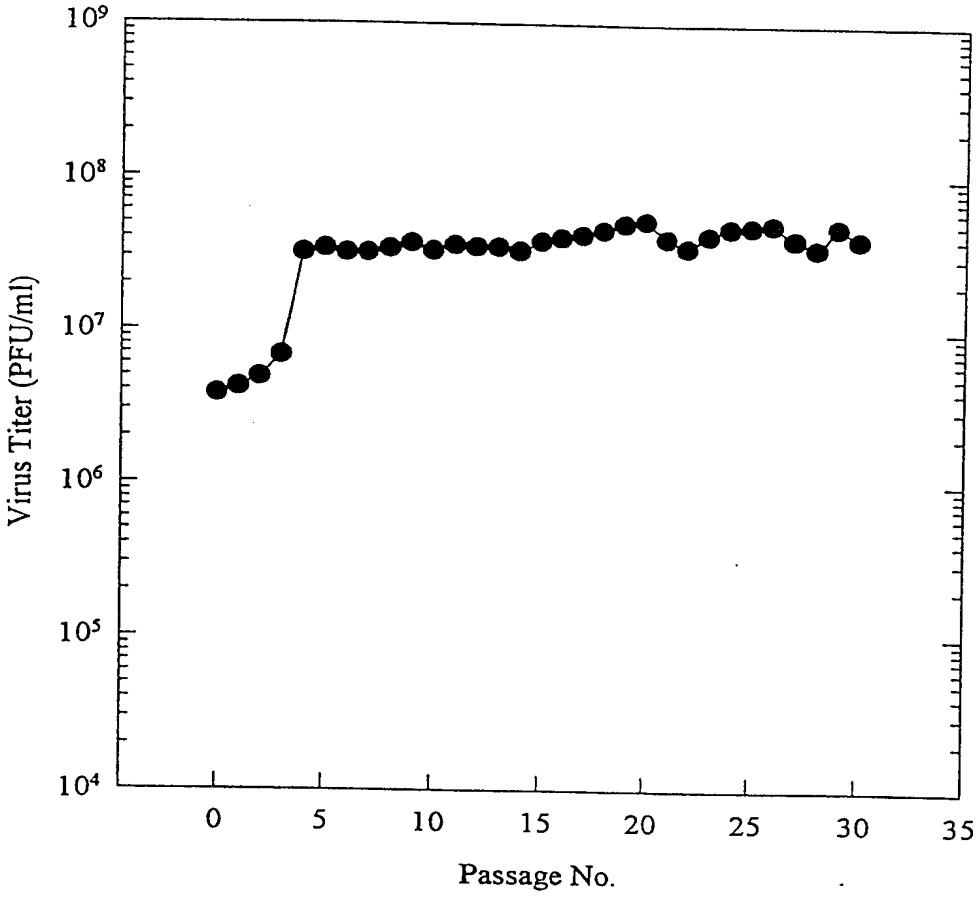

Virus Titer (PFU/ml)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
0
5
10
15
20
25
30
35
Passage No.

Fig 2.

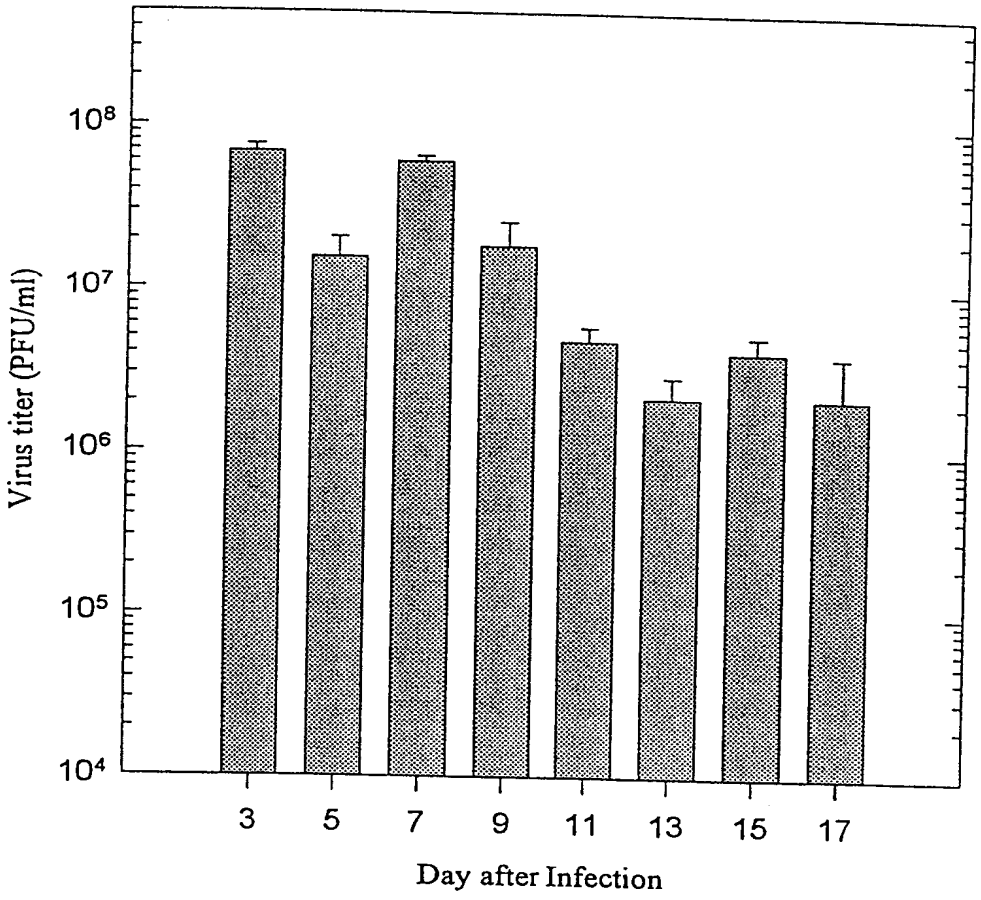
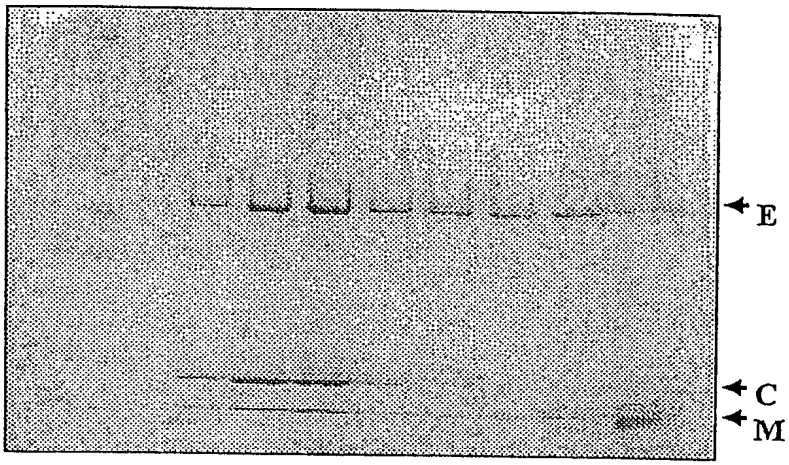
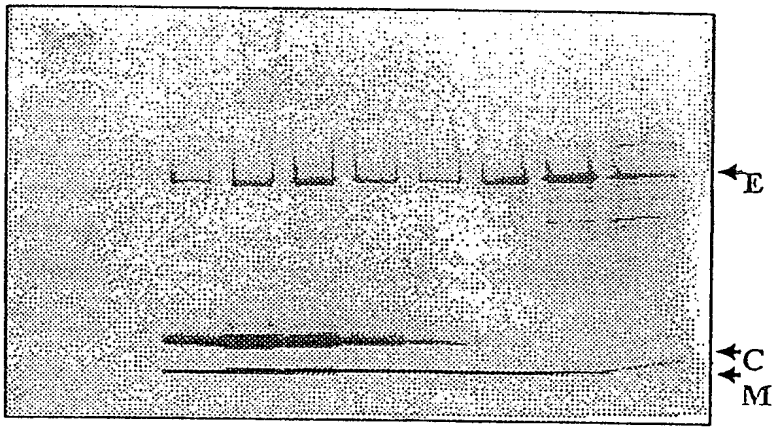

Virus titer (PFU/ml)
10⁸
10⁷
10⁶
10⁵
10⁴
3
5
7
9
11
13
15
17
Day after Infection

Fig 3.

A



B



C

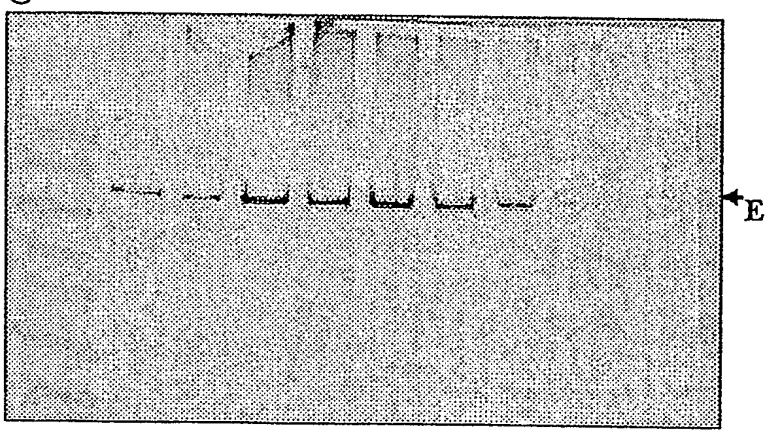
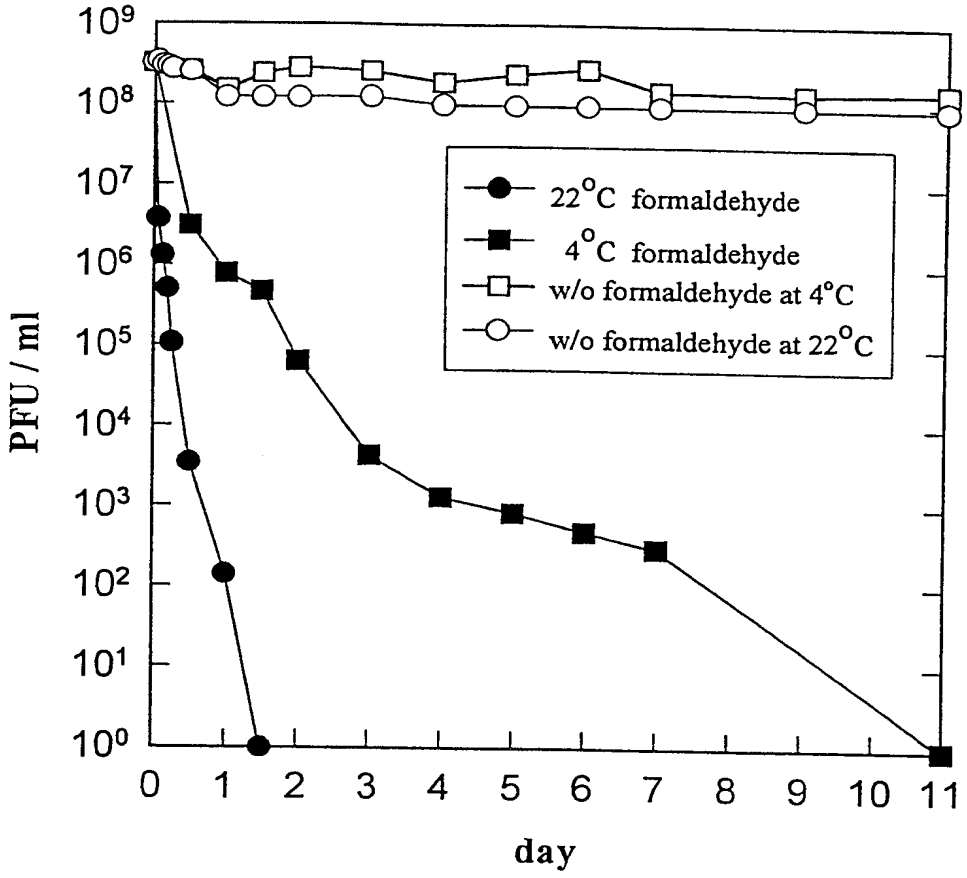


Fig 4.


PFU / ml
day
22°C formaldehyde
4°C formaldehyde
w/o formaldehyde at 4°C
w/o formaldehyde at 22°C

INTERNATIONAL SEARCH REPORT

Inter onal application No.
PCT/KR 98/00259

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 7/00,7/08; A 61 K 39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 7/00,7/08; A 61 K 39/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, Vol.14, No.535, 1990, JP 2-223531 A (TEKADA CHEM. IND., LTD.) 26 November 1990 (26.11.90), abstract.	1,4
A	EP 0 562 136 A1 (DIVISION OF MICROBIOLOGY, KYOTO BIKEN LABORATORIES, INC.) 29 September 1993 (29.09.93), abstract.	1,4
A	Patent Abstracts of Japan, Vol.13, No.339, 1989, JP 1-117780 A (NIPPON ZOKI PHARMACEUT CO., LTD.) 31 July 1989 (31.07.89), abstract.	1,4

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

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Inter. onal application No.
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In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A1 562136	29-09-93	keine - none - rien	

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(54) Title: METHOD FOR THE PURIFICATION OF ALPHAVIRUS REPLICON PARTICLES

(57) Abstract: Methods of production and purification for viruses and virus-derived vectors, including those related to alphaviruses, are disclosed. In one aspect, methods of purification that subject alphavirus replicon particle preparations to one or more steps of chromatographic purification, such as using an ion exchange resin, are provided. Also disclosed are methods of characterizing alphavirus replicon particles and utilizing these materials for vaccines and gene-based therapeutics.

METHOD FOR THE PURIFICATION OF ALPHAVIRUS REPLICON PARTICLES

Technical Field of the Invention

The present invention relates generally to the purification of viruses and virus-derived vectors, including those related to alphaviruses, from biological and chemical preparations. In particular, this invention relates to methods of purification of such viruses and vectors from preparations by subjecting the preparation to chromatographic purification using an ion exchange resin or combination of an ion exchange resin step and another chromatographic process step such as size exclusion or affinity chromatography. The method provides purified viruses and vectors for use as effective vaccines and therapeutics. Moreover related methods for quantifying replicon vector preparations and verifying the replication incompetency of purified vectors are provided.

Background of the Invention

Alphaviruses comprise a set of genetically, structurally, and serologically related arthropod-borne viruses of the *Togaviridae* family. Twenty-six known viruses and virus subtypes have been classified within the alphavirus genus, including, Sindbis virus, Semliki Forest virus, Ross River virus, and Venezuelan equine encephalitis virus.

Sindbis virus is the prototype member of the *Alphavirus* genus of the *Togaviridae* family. Its replication strategy has been well characterized in a variety of cultured cells and serves as a well-accepted model for other alphaviruses. Briefly, the genome of Sindbis virus (like other alphaviruses) is an approximately 12 kb single-stranded positive-sense RNA molecule which is capped and polyadenylated, and contained within a virus-encoded capsid protein shell. The nucleocapsid is further surrounded by a host-derived lipid envelope into which two viral-specific glycoproteins, E1 and E2, are inserted and anchored to the nucleocapsid. Certain alphaviruses (*e.g.*, SFV) also maintain an additional protein, E3, which is a cleavage product of the E2 precursor protein, PE2.

After virus particle adsorption to target cells, penetration, and uncoating of the nucleocapsid to release viral genomic RNA into the cytoplasm, the replicative process occurs via four alphaviral nonstructural proteins (nsPs), translated from the 5' two-thirds of the viral genome. Synthesis of a full-length negative strand RNA, in turn, provides template for the synthesis of additional positive strand genomic RNA and an abundantly expressed 26S subgenomic RNA, initiated internally at the junction region promoter. The alphavirus structural

proteins (sPs) are translated from the subgenomic 26S RNA, which represents the 3' one-third of the genome, and like the nsPs, are processed post-translationally into the individual proteins.

Several members of the alphavirus genus are being developed as "replicon" expression vectors for use as vaccines and therapeutics. Replicon vectors may be utilized in several formats, including DNA, RNA, and recombinant vector particles. Such replicon vectors have been derived from alphaviruses that include, for example, Sindbis virus (Xiong et al., *Science 243*:1188-1191, 1989; Dubensky et al., *J. Virol. 70*:508-519, 1996; Hariharan et al., *J. Virol. 72*:950-958, 1988; Polo et al., *PNAS 96*:4598-4603, 1999), Semliki Forest virus (Liljestrom, *Bio/Technology 9*:1356-1361, 1991; Berglund et al., *Nat. Biotech. 16*:562-565, 1998), and Venezuelan equine encephalitis virus (Pushko et al., *Virology 239*:389-401, 1997). A wide body of literature has now demonstrated efficacy of such replicon vectors for applications such as vaccines (see for example, Dubensky et al., *ibid*; Berglund et al., *ibid*; Hariharan et al., *ibid*, Pushko et al., *ibid*; Polo et al., *ibid*; Davis et al., *J Virol. 74*:371-378, 2000; Schlesinger and Dubensky, *Curr Opin. Biotechnol. 10*:434-439, 1999; Berglund et al., *Vaccine 17*:497-507, 1999).

Because of their configuration, vector replicons do not express the alphavirus structural proteins necessary for packaging into recombinant alphavirus particles (replicon particles). Thus, to generate replicon particles, these proteins must be provided in *trans*. Packaging may be accomplished by a variety of methods, including transient approaches such as co-transfection of *in vitro* transcribed replicon and defective helper RNA(s) (Liljestrom, *Bio/Technology 9*:1356-1361, 1991; Bredenbeek et al., *J. Virol. 67*:6439-6446, 1993; Frolov et al., *J. Virol. 71*:2819-2829, 1997; Pushko et al., *Virology 239*:389-401, 1997; U.S. Patents 5,789,245 and 5,842,723) or plasmid DNA-based replicon and defective helper constructs (Dubensky et al., *J. Virol. 70*:508-519, 1996), as well as introduction of alphavirus replicons into stable packaging cell lines (PCL) (Polo et al., *PNAS 96*:4598-4603, 1999; U.S. Patents 5,789,245, 5,842,723, and 6,015,694; PCT publications WO 9738087 and WO 9918226).

Alphavirus replicon particles produced using any of the above methodologies subsequently are harvested in the cell culture supernatants. The replicon particles then may be concentrated and partially purified using one of several published approaches, including polyethylene glycol (PEG) precipitation, ultracentrifugation, or Cellufine sulfate™ ion exchange chromatography. Unfortunately, these methods do not remove a sufficient level of non-alphavirus derived protein contaminants, are not scalable, or are costly, and therefore are likely not amenable for commercial manufacture necessary of vaccine and therapeutic products.

The present invention provides methods of production and purification with utility for the large-scale manufacture of alphavirus replicon particles. Also disclosed are novel methods for quantitating vector particles in a preparation and determining the presence or absence of contaminating replication-competent virus in a preparation. Additional methods are provided for detecting the presence of packaged helper RNAs in a preparation of replicon particles. Alphavirus particles produced and characterized according to the methods described herein may be used for a variety of applications, including for example, vaccines and gene therapy.

Summary of the Invention

Briefly stated, the present invention provides methods of production and purification for alphavirus replicon particles. Such replicon particles may be derived from a wide variety of alphaviruses (*e.g.*, Semliki Forest virus, Ross River virus, Venezuelan equine encephalitis virus, Sindbis virus), and are designed to express a variety of heterologous proteins (*e.g.*, antigens, immunostimulatory proteins, therapeutic proteins).

Within one aspect of the invention, a method of purifying alphavirus replicon particles is provided. Purification is achieved by first contacting a preparation containing alphavirus replicon particles with an ion exchange resin, under conditions and for a time sufficient to bind to the resin. Next, the portion of the preparation which is not bound to the ion exchange resin is removed from the ion exchange resin, and then the bound alphavirus replicon particles are eluted from the ion exchange resin and recovered. In one embodiment, the ion exchange resin is a tentacle ion exchange resin. In another embodiment, the tentacle ion exchange resin is a cationic exchange resin. In yet another embodiment, the tentacle ion exchange resin is an anionic exchange resin.

Within another aspect of the invention, a method of purification for alphavirus replicon particles is provided, comprising at least two chromatographic purification steps. The chromatographic purification steps are selected from the group consisting of ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, and affinity chromatography. In one preferred embodiment, purification is performed using a first step of ion exchange chromatography and a second step of size exclusion chromatography.

Within another aspect of the invention, a method of producing alphavirus replicon particles is provided. Alphavirus packaging cells are infected with a seed stock of alphavirus replicon particles and then incubated in a bioreactor, under conditions and for a time sufficient to permit the production of alphavirus replicon particles. Next the culture supernatants

containing the replicon particles are harvested. In one embodiment, the bioreactor is an external component bioreactor. In another embodiment the bioreactor is a suspension culture bioreactor.

Within another aspect of the invention, a method of producing alphavirus replicon particles is provided. Alphavirus packaging cells are transfected with a DNA-based alphavirus replicon or eukaryotic layered vector initiation system and then incubated in a bioreactor, under conditions and for a time sufficient to permit the production of alphavirus replicon particles. Next the culture supernatants containing the replicon particles are harvested.

Within another aspect of the invention, a method of producing alphavirus replicon particles is provided wherein alphavirus packaging cells are transfected with an alphavirus RNA vector replicon transcribed *in vitro* and then incubated in a bioreactor, under conditions and for a time sufficient to permit the production of alphavirus replicon particles. Next the culture supernatants containing the replicon particles are harvested.

Within other aspects of the invention, methods of generating alphavirus replicon particles for use in vaccine or therapeutic applications are provided. Replicon particles are produced in packaging cell lines and purified by a chromatographic purification process as described above. In preferred embodiments, the chromatographic purification process includes a step of ion exchange chromatography using a tentacle ion exchange resin.

Within yet other aspects of the present invention, a preparation of alphavirus replicon particles is provided, wherein the preparation of replicon particles is purified by a chromatographic purification process as described above. In preferred embodiments, the chromatographic purification process includes a step of ion exchange chromatography using a tentacle ion exchange resin.

Within a related aspect, a vaccine or immunogenic composition comprising a preparation of alphavirus replicon particles purified by a chromatographic purification process as described above is provided. The preparation of replicon particles being capable of expressing an antigen derived from a pathogenic agent. In preferred embodiments, the chromatographic purification process includes a step of ion exchange chromatography using a tentacle ion exchange resin. In one embodiment, the antigen is derived from a tumor cell. In another embodiment, the antigen is derived from an infectious agent (*e.g.*, virus, bacteria, fungus, and parasite). In preferred embodiments, the antigen is derived from HIV (e.g. gaga, gp120, gp140, gp160, pol, rev, tat, and nef) or HCV (e.g. C, E1, E2, NS3, NS4, and NS5).

Within yet other related aspects, methods for stimulating an immune response within a warm-blooded animal, comprising the step of administering to a warm-blooded animal a

preparation of alphavirus replicon particles purified by a chromatographic purification process as described above are provided, the preparation of replicon particles being capable of expressing an antigen derived from a pathogenic agent. In preferred embodiments, the chromatographic purification process includes a step of ion exchange chromatography using a tentacle ion exchange resin. In one embodiment, the antigen is derived from a tumor cell. In another embodiment, the antigen is derived from an infectious agent (*e.g.*, virus, bacteria, fungus, parasite). In preferred embodiments, the antigen is derived from HIV or HCV.

Within yet other related aspects, methods for stimulating an immune response within a warm-blooded animal, comprising the step of administering to a warm-blooded animal a preparation of alphavirus replicon particles purified by a chromatographic purification process as described above are provided, the preparation of replicon particles being capable of expressing a lymphokine, cytokine, or chemokine. In preferred embodiments, the chromatographic purification process includes a step of ion exchange chromatography using a tentacle ion exchange resin. In one embodiment, the lymphokine, cytokine or chemokine is selected from the group consisting of IL-2, IL-10, IL-12, gamma interferon, GM-CSF, MIP3α, MIP3β, and SLC.

Still other embodiments of the present invention provide for techniques used to establish vector particle preparation safety and potency. One important aspect of vector particle safety is that the preparation be free of contaminating replication-competent alphaviral particles. The packaging cell lines used to produce the vector particles of the present invention contain at least three separate nucleic acid sources used to produce the vector particles of the present invention. One nucleic acid source contains nonstructural viral proteins and a gene of interest, another contains genes encoding for structural proteins and a third encodes for structural proteins not present in any other nucleic acid source. Therefore, contaminating replication-competent alphaviral particles can only arise if a minimum of two recombination events occur.

In one embodiment a preparation of replicon particles free from detectable contaminating replication-competent alphaviral particles is assured using polymerase chain reaction (PCR) techniques wherein a nucleic acid substrate suitable for detecting multiple recombination events is provided. The substrate is derived from a population of alphavirus replicon particles and the nucleic acid substrate is reacted with at least one first reaction mixture comprising an oligonucleotide complementary to an alphavirus nonstructural protein gene and an oligonucleotide complementary to an alphavirus structural protein gene. The structural protein gene is either a capsid protein gene or a non-capsid structural protein gene. Suitable

reaction conditions and time are provided to permit amplification of the nucleic acid substrate and the formation of a first reaction product. Next, the first reaction product is reacted with a second reaction mixture containing an oligonucleotide complementary to an alphavirus capsid protein gene and an oligonucleotide complementary to a non-capsid alphavirus structural protein gene. Suitable reaction conditions and time are provided to permit amplification of the nucleic acid substrate and the formation of a second reaction product. After the first and second reactions are complete, the presence or absence of the second reaction product is established.

In another embodiment of the present invention multiple recombination events are detected by providing a nucleic acid substrate suitable for detecting multiple recombination events, the substrate being derived from a population of alphavirus replicon particles. Then reacting the nucleic acid substrate with a first reaction mixture comprising an oligonucleotide complementary to an alphavirus nonstructural protein gene and an oligonucleotide complementary to an alphavirus capsid protein gene. Conditions suitable and for a time sufficient to permit amplification of the nucleic acid substrate to form a first reaction product are provided. Next, the first reaction product is reacted with a second reaction mixture comprising an oligonucleotide complementary to an alphavirus capsid protein gene and an oligonucleotide complementary to a non-capsid alphavirus structural protein gene. Again, under conditions suitable and for a time sufficient to permit amplification of the nucleic acid template to form a second reaction product. Finally, determining the presence or absence of the second reaction product

In yet another embodiment a method for detecting multiple recombination events is provided comprising providing a nucleic acid substrate suitable for detecting multiple recombination events. The substrate is derived from a population of alphavirus replicon particles and then reacting the nucleic acid substrate with a first reaction mixture comprising an oligonucleotide complementary to an alphavirus nonstructural protein gene and an oligonucleotide complementary to a non-capsid alphavirus structural protein gene. Suitable reaction conditions and time are provided to permit amplification of the nucleic acid substrate to form a first reaction product. Next the first reaction product is reacted with a second reaction mixture comprising an oligonucleotide complementary to an alphavirus capsid protein gene and an oligonucleotide complementary to a non-capsid alphavirus structural protein gene. After a suitable incubation time, the presence or absence of the second reaction product is determined.

In one preferred embodiment, at least two of the above methods for detecting multiple recombination events are performed using the same nucleic acid substrate derived from a population of alphavirus replicon particles.

In another embodiment of the present invention replicon particle preparation potency is quantified. In this embodiment, methods are provided for quantitating or "titering" replication incompetent RNA virus vector particles in a sample. The methods comprising providing a population of packaging cells, contacting the packaging cells with the sample under conditions suitable and for a time sufficient for the cells to be infected with replication-incompetent virus vector particles. Then incubating the infected packaging cells under conditions suitable and for a time sufficient for production of virus vector particles and enumerating the number of resulting plaques.

These and other aspects and embodiments of the invention will become evident upon reference to the following detailed description and attached figures. In addition, various references are set forth herein that describe in more detail certain procedures or compositions (*e.g.*, plasmids, sequences, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

Brief Description of the Figures

Figure 1 is a schematic illustration of an alphavirus replicon packaging cell line with a split structural protein gene expression cassette configuration.

Figure 2 is a graph showing the production of alphavirus replicon particles using packaging cell line #15-25, in a 10 layer Cell Factory.

Figure 3 is a schematic illustration of a CellCube™ bioreactor system

Figure 4 is a graph showing the scale-up production of 90 liters of alphavirus replicon particles using early generation packaging cell lines in a 100-stack CellCube™ system.

Figure 5 is a graph comparing the purification of alphavirus replicon particles using two different single-step methods of ion exchange chromatography.

Figure 6 are Coomassie stained protein gels comparing the purification of alphavirus replicon particles using two different single-step methods of ion exchange chromatography.

Figure 7 is a graph showing the purification of alphavirus replicon particles using the tentacle cationic exchange resin s-Fractogel®.

Figure 8 are Coomassie and silver stained protein gels showing the purification of alphavirus replicon particles using a two step chromatographic process.

Figure 9 is a graph showing the induction of HIV gag antigen specific T cells using alphavirus replicon particles subjected to PEG precipitation or single-step Fractogel chromatographic purification or two-step Fractogel/S-400 chromatographic purification.

Figure 10 is a graph showing the anti-tumor effect of SIN alphavirus replicon particles expressing IL2 in a CT26 colon carcinoma model, as compared to recombinant IL-2 protein or SIN replicon particles expressing GFP reporter.

Figure 11 is a graph showing the use of bDNA assay for detection and quantitation of replicon RNA in a preparation of alphavirus replicon particles as a means to determine titer.

Figure 12 is a schematic illustration of a method for detection of multiple recombination events using nucleic acid amplification to determine the presence or absence of contaminating replication-competent virus in a preparation of alphavirus replicon particles

Definition of Terms

The following terms are used throughout the specification. Unless otherwise indicated, these terms are defined as follows:

"Alphavirus RNA vector replicon", "RNA vector replicon" and "replicon" refers to an RNA molecule which is capable of directing its own amplification or self-replication *in vivo*, within a target cell. To direct its own amplification, the RNA molecule should encode the polymerase(s) necessary to catalyze RNA amplification (*e.g.*, nsP1, nsP2, nsP3, nsP4) and contain *cis* RNA sequences required for replication which are recognized and utilized by the encoded polymerase(s). An alphavirus RNA vector replicon should contain the following ordered elements: 5' viral sequences required in *cis* for replication (also referred to as 5' CSE), sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), 3' viral sequences required in *cis* for replication (also referred to as 3' CSE), and a polyadenylate tract. The alphavirus RNA vector replicon also may contain a viral subgenomic "junction region" promoter, which may, in certain embodiments, be modified in order to increase or reduce viral transcription of the subgenomic fragment, and heterologous sequence(s) to be expressed.

"Recombinant Alphavirus Particle", "Alphavirus replicon particle" and "Replicon particle" refers to a virion unit containing an alphavirus RNA vector replicon. Generally, the recombinant alphavirus particle comprises one or more alphavirus structural proteins, a lipid envelope and an RNA vector replicon. Preferably, the recombinant alphavirus particle contains a nucleocapsid structure that is contained within a host cell-derived lipid bilayer, such as a

plasma membrane, in which one or more alphaviral envelope glycoproteins are embedded. The particle may also contain other components (*e.g.*, targeting elements such as biotin, other viral structural proteins, hybrid envelopes, or other receptor binding ligands) which direct the tropism of the particle from which the alphavirus was derived.

"Alphavirus packaging cell line" refers to a cell which contains one or more alphavirus structural protein expression cassettes and which produces recombinant alphavirus particles after introduction of an alphavirus RNA vector replicon, eukaryotic layered vector initiation system, or recombinant alphavirus particle. The parental cell may be of mammalian or non-mammalian origin. Within preferred embodiments, the packaging cell line is stably transformed with the structural protein expression cassette(s).

"Eukaryotic Layered Vector Initiation System" refers to an assembly that is capable of directing the expression of a sequence or gene of interest. The eukaryotic layered vector initiation system should contain a 5' promoter which is capable of initiating *in vivo* (*i.e.* within a eukaryotic cell) the synthesis of RNA from cDNA, and a nucleic acid vector sequence (*e.g.*, viral vector) which is capable of directing its own replication in a eukaryotic cell and also expressing a heterologous sequence. Preferably, the nucleic acid vector sequence is an alphavirus-derived sequence and is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5' viral sequences required in *cis* for replication or 5' CSE), as well as sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), and an alphavirus RNA polymerase recognition sequence (also referred to as 3' viral sequences required in *cis* for replication or 3' CSE). In addition, the vector sequence may include an alphaviral subgenomic "junction region" promoter which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the subgenomic fragment, as well as a polyadenylation sequence. The eukaryotic layered vector initiation system may also contain splice recognition sequences, a catalytic ribozyme processing sequence, a nuclear export signal, heterologous gene, and a transcription termination sequence. In certain embodiments, *in vivo* synthesis of the vector nucleic acid sequence from cDNA may be regulated by the use of an inducible promoter or subgenomic expression may be inducible through the use of translational regulators or modified nonstructural proteins.

"External Component Bioreactor" refers to an integrated modular bioreactor system for the mass culture, growth, and process control of substrate attached cells. The External Component Bioreactor should have a vessel or chamber with tissue culture treated growth

surface for attachment and propagation of cells (*e.g.*, alphavirus packaging cells). Unlike traditional "stir-tank" bioreactors, which may have an internal mechanical agitation (*e.g.*, impeller) and/or sparging system to circulate culture media and facilitate gas exchange, the External Component Bioreactor should have external components or modules that are connected (*i.e.*, via tubing), to achieve similar functions. In certain embodiments, the external components may include pumps, reservoirs, oxygenators, culture modules, and other non-standard parts.

"Tentacle ion exchange resin" refers to a resin, gel or matrix with functional charge groups and wherein the functional charge groups are carried by long polymer chains ("tentacles"), rather than being located on the surface of the resin, gel or matrix. In certain embodiments, the tentacle ion exchange resin is a cationic resin, gel or matrix that may be used to bind and fractionate biological substances on the basis of charge characteristics. A representative example of a tentacle cationic exchange resin is Fractogel® EMD SO_3^- (M) (s-Fractogel®). In other embodiments, the tentacle ion exchange resin is an anionic resin, gel or matrix that may be used to bind and fractionate biological substances on the basis of charge characteristics. A representative example of a tentacle anionic exchange resin is Fractogel® EMD DEAE (M).

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description, which provides illumination of the practice of the invention.

Detailed Description of the Invention

As noted above, the present invention provides methods of purification for viruses and virus-derived vectors, including those related to alphaviruses, from biological and chemical preparations. In particular, this invention relates to methods of purification for such viruses and vectors from preparations by subjecting the preparation to chromatographic purification methods, such as for example, using a tentacle cationic exchange resin with or without a second chromatographic purification step. Viruses and vectors purified according to this invention have use as effective vaccines and therapeutics.

A. Alphavirus Vector Replicons and Replicon Particles

As noted above, the present invention provides replicons and replicon particles derived from a wide variety of alphaviruses. Such replicons and particles, including sequences encoding

alphaviruses suitable for use in preparing the above-described materials, have been described in detail elsewhere (*see*, for example, U.S. Patent Nos. 5,789,245, 5,842,723, and 6,015,694; PCT Nos. WO 97/38087, WO 99/18226, WO 00/61772, and WO 00/39318), which are hereby incorporated by reference in their entirety.

B. Heterologous Sequences

A wide variety of nucleotide sequences may be carried and expressed by the alphavirus replicon particles of the present invention, including, for example, sequences which encode lymphokines, cytokines, or chemokines (*e.g.*, IL-2, IL-12, GM-CSF, SLC), prodrug converting enzymes (*e.g.*, HSV-TK, VZV-TK), antigens which stimulate an immune response (*e.g.*, HIV, HCV, tumor antigens), therapeutic molecules such as growth or regulatory factors (*e.g.*, VEGF, FGF, PDGF, BMP), proteins which assist or inhibit an immune response, as well as ribozymes and antisense sequences. The above nucleotide sequences include those referenced previously (*e.g.*, U.S. 6,015,686, WO 97/38087 and WO 99/18226, WO 00/61772, and WO 00/39318), and may be obtained from repositories, readily cloned from cellular or other RNA using published sequences, or synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, CA)).

C. Alphavirus Replicon Particle Production

Alphavirus replicon particles according to the present invention may be produced using a variety of published methods. Such methods include, for example, transient packaging approaches, such as the co-transfection of *in vitro* transcribed replicon and defective helper RNA(s) (Liljestrom, *Bio/Technology 9*:1356-1361, 1991; Bredenbeek et al., *J. Virol. 67*:6439-6446, 1993; Frolov et al., *J. Virol. 71*:2819-2829, 1997; Pushko et al., *Virology 239*:389-401, 1997; U.S. Patents 5,789,245 and 5,842,723) or plasmid DNA-based replicon and defective helper constructs (Dubensky et al., *J. Virol. 70*:508-519, 1996), as well as introduction of alphavirus replicons into stable packaging cell lines (PCL) (Polo et al., *PNAS 96*:4598-4603, 1999; U.S. Patents 5,789,245, 5,842,723, 6,015,694; WO 97/38087, WO 99/18226, WO 00/61772, and WO 00/39318).

It should be noted that the selected method for production of replicon particles should preferably minimize or eliminate the possibility of generating contaminating replication-competent virus (RCV). One such strategy to address this issue of RCV is the use of defective helpers or PCL that contain "split" structural protein expression cassettes (*see* U.S. Patent

5,789,245). In this context, the alphavirus structural protein genes are segregated into separate expression constructs (*e.g.*, capsid separate from glycoproteins) such that multiple recombination events are required to regenerate a complete complement of structural proteins, which is extremely unlikely.

In preferred embodiments, stable alphavirus packaging cell lines are utilized for replicon particle production (Figure 1). The PCL may be transfected with *in vitro* transcribed replicon RNA, transfected with plasmid DNA-based replicon (*e.g.*, ELVIS vector), or infected with a seed stock of replicon particles, and then incubated under conditions and for a time sufficient to produce high titer packaged replicon particles in the culture supernatant. In particularly preferred embodiments, PCL are utilized in a two-step process, wherein as a first step, a seed stock of replicon particles is produced by transfecting the PCL with a plasmid DNA-based replicon. A much larger stock of replicon particles is then produced in the second step, by infecting a fresh culture of the PCL with the seed stock. This infection may be performed using various multiplicities of infection (MOI), including a MOI = 0.01, 0.05, 0.1, 0.5, 1.0, 3, 5, or 10. Preferably infection is performed at a low MOI (*e.g.*, less than 1). Replicon particles at titers $>10^8$ infectious units (IU)/ml can be harvested over time from PCL infected with the seed stock. In addition, the replicon particles can subsequently be passaged in yet larger cultures of naïve PCL by repeated low multiplicity infection, resulting in commercial scale preparations with the same high titer. Importantly, by using PCL of the "split" structural gene configuration, these replicon particle stocks are free from detectable contaminating RCV.

As described above, large-scale production of alphavirus replicon particles may be performed using a bioreactor. Preferably, the bioreactor is an External Component Bioreactor, which is an integrated modular bioreactor system for the mass culture, growth, and process control of substrate attached cells. The attachment and propagation of cells (*e.g.*, alphavirus packaging cells) occurs in a vessel or chamber with tissue culture treated surfaces, and the cells are with fresh media for increased cell productivity. Monitoring and adjustments are performed for such parameters as gases, temperature, pH, glucose, etc., and crude vector is harvested using a perfusion pump. Typically, the individual components of an External Bioreactor separate external modules that are connected (*i.e.*, via tubing). The external components can be pumps, reservoirs, oxygenators, culture modules, and other non-standard parts. A representative example of an External Component Bioreactor is the CellCube™ system (Corning, Inc).

In addition to using the External Component Bioreactor described herein, a more traditional Stir Tank Bioreactor may also be used, in certain instances, for alphavirus replicon particle production. In a Stir Tank Bioreactor, the alphavirus packaging cells may be unattached to any matrix (*i.e.*, floating in suspension) or attached to a matrix (e.g., poly disks, micro- or macro carriers, beads). Alternatively, a Hollow Fiber Culture System may be used.

D. Purification using Ion Exchange Resins

Following harvest, crude culture supernatants containing the alphavirus replicon particles may be clarified by passing the harvest through a filter (*e.g.*, 0.2 uM, 0.45 uM, 0.65 uM, 0.8 uM pore size). Optionally, the crude supernatants may be subjected to low speed centrifugation prior to filtration to remove large cell debris. Within one embodiment, an endonuclease (*e.g.*, Benzonase, Sigma #E8263) is added to the preparation of alphavirus replicon particles before or after a chromatographic purification step to digest exogenous nucleic acid. Further, the preparation may be concentrated prior to purification using one of any widely known methods (*e.g.*, tangential flow filtration).

Crude or clarified alphavirus replicon particles may be concentrated and purified by chromatographic techniques (*e.g.*, ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, affinity chromatography). Two or more such purification methods may be performed sequentially. In preferred embodiments, at least one step of ion exchange chromatography is performed and utilizes a tentacle ion exchange resin. Briefly, clarified alphavirus replicon particle filtrates may be loaded onto a column containing a charged ion exchange matrix or resin (*e.g.*, cation or anion exchange). The matrix or resin may consist of a variety of substances, including but not limited to cross linked agarose, cross linked polystyrene, cross linked styrene, hydrophilic polyether resin, acrylic resin, and methacrylate based resin. The ion exchanger component may comprise, but is not limited to, a cationic exchanger selected from the list consisting of sulphopropyl cation exchanger, a carboxymethyl cation exchanger, a sulfonic acid exchanger, a methyl sulfonate cation exchanger, and an SO3– exchanger. In other embodiments, the ion exchanger component may comprise, but is not limited to, an anionic exchanger selected from the list consisting of DEAE, TMAE, and DMAE. Most preferably, ion exchange chromatography is performed using a tentacle cationic exchanger, wherein the ion exchange resin is a methacrylate-based resin with an SO3– cation exchanger (*e.g.*, Fractogel® EDM SO3-).

The replicon particles may be bound to the ion exchange resin followed by one or more washes with buffer containing a salt (*e.g.*, 250 mM or less NaCl). Replicon particles then may be eluted from the column in purified form using a buffer with increased salt concentration. In preferred embodiments, the salt concentration is a least 300 mM, 350 mM, 400 mM, 450 mM or 500 mM. Elution may be monitored preferably by a spectrophotometer at 280 nm, but also by replicon titer assay, transfer of expression (TOE) assay, or protein gel analysis with subsequent Coomassie staining or Western blotting.

The higher salt elution buffer subsequently may be exchanged for a more desirable buffer, for example, by dilution in the appropriate aqueous solution or by passing the particle-containing eluate over a molecular exclusion column. Additionally, the use of a molecular size exclusion column may also provide, in certain instances, further purification. For example, in one embodiment Sephacryl S-500 or S-400 (Pharmacia) chromatography may be used as both a buffer exchange as well as to further purify the fractions containing the replicon particles eluted from an ion exchange column. Using this particular resin, the replicon particles generally are eluted in the late void volume and show improvement in the level of purity as some of the contaminants are smaller in molecular weight and are retained on the column longer. However, alternative resins of different compositions as well as size exclusion could also be used that might yield similar or improved results. In these strategies, larger-sized resins such as Sephacryl S-1000 could be incorporated that would allow the replicon particles to enter into the matrix and thus be retained longer, allowing fractionation.

E. Methods of Determining Replicon Particle Titer

Two methods of titering alphavirus replicon particles are widely accepted in the viral vector field. The first method of titering is a simple transfer of expression assay, wherein a culture of naïve cells is infected with various dilutions (*e.g.*, serial dilutions) of the unknown replicon particle preparation and individual cells expressing the encoded gene of interest are quantitated to arrive at original titer. Identification of cells expressing the encoded gene of interest may be performed according to the specific protein being expressed (*e.g.*, fluorescence for GFP reporter, chemical staining for β-gal, immunocytochemistry for proteins with available antibody). Alternatively, an alphavirus reporter cell line (*e.g.*, Olivo et al., *Virology 198*:381-384, 1984) may be used in conjunction with replicon particles expressing a reporter gene, which serve as a known titer standard curve. Values for the unknown, obtained after infection of the reporter cell line with various dilutions, can be extrapolated to calculate titer.

The present invention discloses additional methods of quantitating replicon particles in a preparation, and these methods are not limited on a preparation by preparation basis, such as from one gene of interest to another. The first method is based upon nucleic acid detection and amplification of the nucleic acid product or a signal that is specific to the assay. Such methods can include, for example, PCR, TMA and bDNA (branched DNA) assays. These nucleic acid based assays provide extremely sensitive levels of detection. More specifically, in the case of a bDNA based assay, a single-stranded DNA probe that is specific and unique to a region of alphavirus genomic and replicon RNA was designed. This probe is bound to the bDNA plate. Target cells that have been infected with serial dilutions of replicon particle preparations are lysed and directly transferred to the bDNA plate. After overnight incubation, the alphavirus genomic RNA hybridizes to the homologous single-stranded DNA probe. The plate is then washed to clear non-specific material, and sequentially incubated with a series of hybridization amplifiers. The signal generated is luminescence-based and can be analyzed in a spectrophotometer. A standard curve can be generated using reporter replicon particle preparations of known titer, for example vector encoding ß-galactosidase or green fluorescent protein. The titer of the unknown sample is determined by extrapolation.

The second method of quantitation is by complementation of the replicon vector so as to allow detection by plaque assay in cultured cells. Replication defective viral vectors, such as alphavirus replicons, which are deleted of one or more genes encoding structural proteins necessary for packaging are considered "suicide vectors" and cannot spread from cell to cell. As such, traditional plaque assay methods of quantitation are impossible. The present invention provides a method of performing plaque assay by using packaging cells which express the necessary structural proteins required for production of progeny particles. The packaging cells used for such an assay may contain one or more structural protein expression cassettes. In the case of alphavirus replicon particles, packaging cells are infected with serial dilutions of replicon particle preparations, overlayed and plaques enumerated.

F. Pharmaceutical Compositions

As noted above, the present invention also provides pharmaceutical compositions comprising purified alphavirus replicon particles in combination with a pharmaceutically acceptable carrier, diluent, or recipient. As used herein, purified shall mean an alphavirus replicon particle preparation free from detectable non-alphavirus proteins. Dectection of non-alphavirus proteins is determined by gel electrophoresis using a sample size of between

approximately 10^8 to 10^9 replicon particles. Gel electrophoreiss methods including, but not limited to polyacrylamide gel electrophoresis (PAGE), disc electrophoresis and SDS-PAGE, followed by standard Coomassie staining. More specifically, "purified" shall mean alphavirus particle preparations subjected to multi-step chromatography purification procedures as disclosed herein. Within certain preferred embodiments, a sufficient amount of formulation buffer is added to the purified replicon particles to form an aqueous suspension. In preferred embodiments, the formulation buffer comprises a saccharide and a buffering component in water, and may also contain one or more amino acids or a high molecular weight structural additive. The formulation buffer is added in sufficient amount to reach a desired final concentration of the constituents and to minimally dilute the replicon particles. The aqueous suspension may then be stored, preferably at -70°C, or immediately dried.

The aqueous suspension can be dried by lyophilization or evaporation at ambient temperature. Briefly, lyophilization involves the steps of cooling the aqueous suspension below the gas transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized replicon particle. Within one embodiment, aliquots of the formulated recombinant virus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (*Cryobiology 18*:414, 1981) is used to lyophilize the formulated replicon particles, preferably from a temperature of -40°C to -45°C. The resulting composition contains less than 10% water by weight of the lyophilized replicon particles. Once lyophilized, the replicon particles are stable and may be stored at -20°C to 25°C, as discussed in more detail below. In the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Within one embodiment, water is removed by a spray-drying process, wherein the aqueous suspension is delivered into a flow of preheated gas, usually which results in the water rapidly evaporating from droplets of the suspension. Once dehydrated, the recombinant virus is stable and may be stored at -20°C to 25°C.

The aqueous solutions used for formulation preferably comprise a saccharide, a buffering component, and water. The solution may also include one or more amino acids and a high molecular weight structural additive. This combination of components acts to preserve the activity of the replicon particles upon freezing and also lyophilization or drying through evaporation. Although a preferred saccharide is lactose, other saccharides may be used, such as

sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. A particularly preferred concentration of lactose is 3%-4% by weight.

The high molecular weight structural additive aids in preventing particle aggregation during freezing and provides structural support in the lyophilized or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 M.W. A preferred high molecular weight structural additive is human serum albumin. However, other substances may also be used, such as hydroxyethyl-cellulose, hydroxymethyl-cellulose, dextran, cellulose, gelatin, or povidone. A particularly preferred concentration of human serum albumin is 0.1% by weight.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. In addition, it is preferable that the aqueous solution contains a neutral salt that is used to adjust the final formulated replicon particles to an appropriate iso-osmotic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride or magnesium chloride. A preferred salt is sodium chloride. The lyophilized or dehydrated replicon particles of the present invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions that bring the final formulation to isotonicity may also be used.

G. Methods for Delivery of Replicon Particles

As noted above, the present invention also provides methods for delivering a selected heterologous sequence to a warm-blooded mammal (*e.g.*, a mammal such as a human or other warm-blooded animal such as a horse, cow, pig, sheep, dog, cat, rat or mouse) for use as a vaccine or therapeutic, comprising the step of administering to the mammal replicon particles purified and/or characterized as described herein. Delivery may be by a variety of routes (*e.g.*, intravenously, intramuscularly, intradermally, intraperitoneally, subcutaneously, orally, intraocularly, intranasally, rectally, intratumorally). In addition, the replicon particles may either be administered directly (*i.e., in vivo*), or to cells which have been removed (*ex vivo*), and subsequently returned to the warm-blooded mammal.

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention and are not meant to limit the scope thereof.

EXAMPLES

EXAMPLE 1

PRODUCTION OF ALPHAVIRUS REPLICON PARTICLES USING A PACKAGING CELL LINE

To demonstrate scalability of replicon particle production in adherent cultures of an alphavirus packaging cell line, experiments were performed in either a 10-tray Nunc Cell Factory or a Corning Cell Cube. For example, 2.5 x 10^8 cells of an alphavirus packaging cell line, PCL #15.25, which expresses human dendritic cell tropic Sindbis structural proteins (Gardner et al., *J. Virol.*, 74:11849-11857, 2000) were suspended in 100 ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin, streptomycin, L-glutamine, and 1% fetal calf serum (FCS). To this suspension, 1.26 x 10^8 SIN replicon particles encoding a GFP reporter (Gardner et al., 2000, *ibid*) were added at a multiplicity of infection (MOI) of approximately 0.57 particles per cell. The suspension was incubated at 37°C and gently mixed every 15 minutes for approximately 1.5 hours. The suspension was then added to 1 liter of pre-warmed (37°C) DMEM with 5% FCS, transferred to a 10-tray Nunc Cell Factory, and placed in an incubator set at 34°C, 5% CO_2. Complete media exchanges were made at 22 hr, 30 hr, 44 hr, 52 hr, 70 hr, 78 hr, and 90 hr post-infection and replicon particle titers were determined for each harvest (Figure 2). Culture fluids collected for the highest titer harvests (harvests 1-5) were pooled, transferred to centrifuge bottles, and cell debris was pelleted by centrifugation at 2,500 RPM in a Sorvall RT6000 centrifuge, at 4°C for 15 minutes. The supernatant then was passed through a 0.2 um cellulose acetate filtration unit and used for chromatographic purification as described in example 2 below. Similar production runs have been performed using more than one Cell Factory, in order to increase the total harvest of alphavirus replicon particles proportionally.

Large-scale manufacture of alphavirus replicon particles also may be accomplished, for example, using the CellCube™ bioreactor system (Figure 3). The CellCube™ system is an integrated modular bioreactor with multi-layer (100-stack) growth chambers of 85,000cm^2 surface area. Controlling the mixtures of oxygen, CO_2 and air allows precise control of pH and

DO_2 parameters. Together with glucose monitoring and adjustment, this level of culture control provides an increased capacity for replicon particle production.

PCL-based production runs of alphavirus replicon particles using the CellCube™ system requires the input of an initial seed stock of replicon particles to be amplified. To demonstrate feasibility of this approach, CellCube™ production was performed by expanding PCL successively in T225cm^2 cell culture flasks, and increasing to a surface area of four 10-layer Cell Factories prior to suspension infection with the particle seed stock. The PCL were trypsinized using a minimal amount of trypsin, diluted with growth media and then centrifuged briefly. Resuspended cells were counted, split into equal halves, and infected with the seed stock of replicon particles at low MOI infection. Infection was allowed to proceed in suspension with gentle agitation for 30 minutes. After 30 minutes, one vessel was placed on ice and the other was transferred into the inoculation carboy containing 7L of 5% FBS DMEM inoculation media. The 7L of infected cell suspension was transferred into the CellCube and the culture module was rotated 90° to enable the cells to attach. After 60 minutes, the suspension was drained back into the inoculation carboy and the second vial of infected PCL (from ice) was added. This suspension, like the first, was transferred into the culture module, which was then rotated 180° to enable these cells to attach to the opposite side of the culture support plates. After 3 hours, the system was rotated back to the horizontal position and back-filled with 5% DMEM, 20mM HEPES, after which, circulation was initiated, and gasses were adjusted to maintain pH and DO2 levels. Daily sampling allowed testing and profiling of metabolic indicators including glucose levels. The perfusion system was adjusted based on glucose consumption and previous data to ensure maximum yield of vector. Automated and continuous harvest into 4°C vessels was used to minimize temperature-induced degradation of replicon particles and allow maximum yield and highest ratio of viable replicon particles.

The initial CellCube™ production runs illustrated for this example were performed using early generation replicon and packaging cell lines (prior to the derivation of PCL #15-25 above). These earlier versions of reagents are known to yield alphavirus replicon particles at lower titers (Polo et al., 1999, *PNAS* 96:4598-4603) than the reagents now available, however such techniques are identical to those that would be used for any subsequently derived vector replicon and packaging cell line. The data obtained using these reagents indicate that large-scale (90-100 liter) production lots can be generated in a 100-stack CellCube™ module, with the same titer efficiency as small, research-scale packaging experiments (Figure 4). Expanded

CellCube™ systems that employ four 100-stack modules, thus, have the potential to readily produce 400L+ of replicon particle production material per run.

To generate seed stocks of alphavirus replicon particles without a prior step of *in vitro* transcription, the packaging cell lines may be transfected with a plasmid DNA-based replicon (Eukaryotic Layered Vector Initiation System) encoding the heterologous gene of interest. Large-scale transfections are carried out in Nunc 10 layer Cell Factories, using the calcium phosphate method according to the following parameters. Packaging cells are plated in the Cell Factory one day prior to transfection at a density of 8×10^4 cells/cm^2. The DNA:calcium phosphate mixture is prepared in a volume of 200 ml, diluted with 1 liter of media and added to the packaging cell line in the Cell Factory. The media is exchanged after 6-8 hr and replicon particle seed stock material is harvested in multiple batches, over a period of 2-3 days. Harvests are pooled, purified, and aliquoted for long-term storage at –80°C. Alphavirus replicon particle seed stock material then may be used for subsequent large-scale amplifications in naïve PCL (*e.g.*, in CellCube bioreactor). Alternative methods of transfecting the plasmid DNA-based replicon also may be substituted readily by one of skill in the art, including but not limited to lipid-mediated transfection and electroporation.

EXAMPLE 2

PURIFICATION OF REPLICON PARTICLES USING S-FRACTOGEL® CATIONIC EXCHANGE RESIN

To compare the efficiency of replicon particle purification using a tentacle cationic exchange resin, Fractogel® EMD SO_3^- (M) (s-Fractogel®, EM Industries), with the ion exchange resin Matrix® Cellufine™ Sulfate (Amicon), columns of the same size were equilibrated with 10 mM sodium phoshate, 125 mM sodium chloride, pH 7.0. Clarified culture supernatants (~260 ml) containing SIN-GFP replicon particles generated as described (Polo et al., *PNAS* 96:4598-4603, 1999) were passed through s-Fractogel® and Cellufine™ Sulfate columns at flow rates of 115 and 75cm/hour respectively. The columns were washed with approximately 20-40 column volumes of buffer containing 10mM sodium phoshate, 250mM sodium chloride, pH 7.0, and bound SIN-GFP replicon particles were eluted in a 20ml, 0.5M-2.0M NaCl linear gradient collected in 1 ml fractions. A final 3 M NaCl rise was then used to remove any remaining replicon particles.

For analysis, consecutive fractions were pooled in pairs starting with fractions 2 and 3, continuing with 4 and 5, etc. Replicon particle titers (total IU) were determined for the

recovered fractions, as well as the starting material, load, and wash (Figure 5). Based on the titer assay results, the 260ml of clarified supernatant starting material contained approximately $2.4x10^{10}$ IU total. Recovery in the main elution peaks from the s-Fractogel® column was 1.3 $x10^{10}$ IU total, or approximately 55% of the load, with almost all (99%) concentrated in pooled fractions 2 and 3. Subsequent purification runs using s-Fractogel indicated an average recovery of 80-90%. In contrast, the total recovery from the Cellufine™ Sulfate column was consistently lower, and for this experiment was approximately 3.0 $x10^{8}$ IU total (or <2%) in the two main fractions, thus resulting in a considerably more dilute product.

Samples also were analyzed for purity by subjecting the collected fractions to polyacrylamide gel electrophoresis (Coomassie staining, Figure 6) and Western blotting (not shown). The results of 10-20% SDS PAGE Coomassie-stained gels indicated an improvement in the purity of the s-Fractogel® peak as compared to the Cellufine™ Sulfate peak (see SIN particle capsid and glycoprotein bands in sample 2). Interestingly, the main peak of recovered particles, found in pooled fractions 2 and 3 from each column, eluted just prior to a considerable peak of contaminants in pooled fraction 4 and 5 from each column. If fraction 4/5 material was excluded from the Cellufine™ Sulfate pooled products due to the increased amount of impurity, it would reduce the effective recovery even further.

In addition to the improved efficiency of purification, the cost basis of the s-Fractogel® is considerably lower than for the Cellufine™ Sulfate. Cost analysis for the resin component only indicates an approximately 3-fold cost decrease with s-Fractogel®, assuming that equal amount of resins could be used. However, the data suggest that there may have been an overloading on the Cellufine™ Sulfate column and that additional resin may be required for equivalent binding capacity. Finally, the reduced flow rate of the Cellufine™ Sulfate column would translate to 50% increase in column run time and thus, another increased cost per run. Taken together, the s-Fractogel® purification method of the present invention provides superior overall utility for large-scale commercial manufacture of alphavirus replicon particles.

In additional experiments, increased volumes of alphavirus replicon particles (*e.g.*, those generated using at least a Cell Factory) also were purified using the s-Fractogel® methodology. For example, a total 25 ml of s-Fractogel® was packed in a Pharmacia AK-26 column and equilibrated with 20 column volumes of buffer (10 mM sodium phosphate, pH 7.0 and 125 mM NaCl) at a linear flow rate of 115 cm/hour. After equilibration, approximately 5.5 liters of culture supernatant containing alphavirus replicon (*see* Example 1) was passed over the column.

The column was washed with approximately 300 ml of wash buffer (10 mM sodium phosphate, pH7.0, 250 mM NaCl), and the particles were eluted in 12 ml fractions by buffer containing 10mM sodium phosphate, pH7.0, 400 mM NaCl.

Determination of recovery and identification of peak fractions containing the alphavirus replicon particles was performed by titer assay in which aliquots from the starting material, the flow through, the wash, and the eluted fractions were serially diluted and used to infect BHK-21 cells. The results from this purification procedure (Figure 7) indicate that the 5.5 liters of harvested supernatant contained replicon particles with a titer of approximately 1.2×10^8 IU/ml and that only a negligible amount of particles was found in either the flow through or the wash. The highest concentration of eluted, purified particles was found in the 2nd and 3rd fractions eluted from the column at a concentration of 1.3×10^{11} and 1.4×10^{10} IU/ml.

EXAMPLE 3

STIMULATION OF THE IMMUNE RESPONSE USING ALPHAVIRUS REPLICON PARTICLES

To demonstrate the potent stimulation of antigen specific immune responses using purified alphavirus replicon particles, the sequence encoding HIV-1 p55gag was inserted into SIN-based replicons. The HIV gag coding sequence was selected from the HIV-1SF2 strain (Sanchez-Pescador, R., et al., *Science 227*(4686):484-492, 1985; Luciw, P.A., et al., U.S. Patent No. 5,156,949, herein incorporated by reference; Luciw, P.A., et al., U.S. Patent No. 5,688,688). These sequences have been used directly or first manipulated to maximize expression of their gene products. For maximization of expression, the HIV-1 codon usage pattern was modified so that the resulting nucleic acid coding sequence was comparable to codon usage found in highly expressed human genes. The HIV codon usage reflects a high content of the nucleotides A or T as third base of the codon-triplet. The effect of the HIV-1 codon usage is a high AT content in the DNA sequence that could result in a decreased translation ability and instability of the mRNA. In comparison, highly expressed human codons prefer the nucleotides G or C as the third base. The gag coding sequence therefore was modified to be comparable to codon usage found in highly expressed human genes.

The DNA fragment for gag first was cloned into the eukaryotic expression vector pCMVKm2, derived from pCMV6a (Chapman et al., *Nuc. Acids Res. 19*:3979-3986, 1991), to generate the construct pCMVKm2.GagMod.SF2. This plasmid was deposited January 18, 1999, with the Chiron Corporation Master Culture Collection, Emeryville, CA, 94662-8097,

and with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209. The HIV gag gene then was subcloned into a SIN replicon vector (SINCR, Gardner et al., *ibid*) for the generation of alphavirus replicon particles by digestion with *EcoR*I, blunt-ending with Klenow and dNTPs, purification with GeneCleanII, and digestion with *Sal*I. The HIV gag-coding fragment then was ligated into the SINCR vector that had been digested with *Not*I, blunt-ended, and digested with *Xho*I. The resulting vector was designated SINCR-gag.

To compare the effective purification as well as demonstrate the maintenance of immunogenicity of column-purified replicon particles, a production run of 4x10-tray Nunc Cell Factories was undertaken. Approximately 2 x 10^9 cells of alphavirus packaging cell line, PCL #15.25, were suspended in 400 ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin, streptomycin, L-glutamine, and 1% fetal calf serum (FCS). To this suspension, 1 x 10^{10} SIN replicon particles encoding HIV p55 Gag were added at a multiplicity of infection (MOI) of approximately 5. The suspension was incubated at 37°C and gently mixed every 15 minutes for approximately 1hour. The suspension was then divided into 4 x 100 ml aliquots and each 100 ml aliquot was added to 1 liter of pre-warmed (37°C) DMEM with 5% FCS, transferred to the 10-tray Nunc Cell Factory, and placed in an incubator set at 34°C, 5% CO_2. Complete media exchanges were made at 20 hr, 28 hr, and 40 hr post-infection. Culture fluids collected from at each harvest was transferred to centrifuge bottles, and cell debris was pelleted by centrifugation at 2,500 RPM in a Sorvall RT6000 centrifuge, at 4°C for 15 minutes and the resulting supernatant was passed through a 0.2 μm cellulose acetate filtration unit. Approximately 8 L of supernatant was loaded onto a 2.6 cm diameter column containing 30 ml of s-Fractogel resin equilibrated with 10 mM sodium phoshate, 125 mM sodium chloride, pH 7.0. The flow rate of 58 cm/hour was used for the first 5 liters and 115 cm/hour for the last 3L. The column was rinsed with 10 mM sodium phosphate, 125 mM sodium chloride, pH 7.0 and was followed by two wash steps containing 10 mM sodium phosphate, 250 mM sodium chloride, pH 7.0 then 10 mM sodium phosphate, 300 mM sodium chloride. The particles were eluted with 10 mM sodium phosphate, 400 mM sodium chloride, pH 7.0. The two peak s-Fractogel fractions (#2 and #3) were pooled and 10 ml of the pool was loaded onto a Sephacryl S-400 HR (Pharmacia) (diameter = 2.6 cm, column volume = 490ml) equilibrated with buffer containing 40mg/ml lactose in PBS. The flow rate was 3.3 ml/minute and each fraction contained 12ml. Samples from the s-Fractogel and the S-400 were analyzed for titer recovery as well as purity by as determined by polyacrylamide gel electrophoresis

Coomassie and silver staining. Based on the titer assay, the 4-cell factory run generated approximately $1x10^{12}$ IU total particles. Approximately $8x10^{11}$ IU were loaded onto the s-Fractogel column and approximately $6x10^{11}$ IU were eluted in the main peak yielding a 75% recovery. From the s-Fractogel peak, $3x10^{11}$ IU were loaded onto the S-400 column, with approximately $1.5x10^{11}$ IU eluted in the main peak resulting in a 50% recovery. The relative purity of the s-Fractogel and the S-400 samples are shown in Figure 8.

In order to determine if the purified SIN replicon particles encoding HIV-p55 maintained immunogenicity, a study was designed to compare the purified particles with an essentially unpurified, but concentrated (polyethelyene glycol precipitation) preparation of particles using an Gag-specific IFN-γ ELISPOT. In the study, mice (5 mice per group) were immunized with SIN-gag replicon particle preparations (10^6 IU/animal) that were PEG precipitated, purified with a single step of cationic exchange chromatography, or a two step process of cationic exchange chromatography followed by size exclusion chromatography. Animals received immunizations at days 0 and 21 with sample collection at days 29 and 30.

To measure the number of Gag-specific IFN-γ secreting cells, an ELISPOT assay was performed. Single-cell suspensions from pooled cervical lymph nodes and spleens from the mice in each group were added onto nitrocellulose or pvdf plates (Millipore) pre-coated with monoclonal rat anti-mouse anti-IFN-γ antibody (Pharmingen) and blocked with complete RPMI medium at pH 7.2, containing 10% fetal calf serum, 5mM Hepes, and antibiotics. Following overnight incubation of cells in the presence of gag-derived p7g peptide, or anti-CD3 (Pharmingen) and anti-CD28 (Pharmingen) as positive control for polyclonal T cell activation, or media only as negative control, the plates were washed and biotinylated anti-IFN-? (Pharmingen) was added in PBS/0.1% BSA/0.02% Tween and incubated at R/T for 2 hours. The plates were washed with P/T and incubated for 1 hr at 37°C with Avidin-peroxidase (Pharmingen) at 1:1000 dilution. The plates were washed with P/T and the spots were visualized by adding DAB in Tris-HCl (pH 7.5) buffer for 30 minutes. The plates were washed with de-ionized H_2O and air-dried. Background spots from negative control (media only) wells were subtracted from wells activated with gag-p7g peptide. The number of spots in positive control wells (polyclonally activated with anti-CD3 and anti-CD28) was 5-10 fold higher than the number of spots in wells activated with gag-p7g peptide. The spots were counted with an in-house developed automated ELISPOT reader using software from Alpha Innotech Corporation (San Leandro, CA).

The results shown in Figure 9 are representative of two independent experiments from two pools of each group expressed as the number of gag-p7g peptide-specific IFN-γ secreting cells per 10^7 mononuclear cells. The results indicate no loss of immunogenicity from either method of purification.

Similarly, the stimulation of an antitumor response was demonstrated in the widely accepted CT26 colon carcinoma system by administering SIN derived alphavirus replicon particles expressing the cytokine IL-2. The IL-2 gene was inserted into the SIN replicon vector following PCR amplification and replicon particles were produced using methods described above. On four successive days following tumor inoculation, mice were injected intratumorally with 10^8 SIN-IL2 replicon particles. Additional animals received as controls the diluent only, recombinant IL2 protein which has an established clinical efficacy in humans, or SIN-GFP particles. Animals were monitored for increased tumor volume and group means for each treatment group arm were compared. When the group mean for a given arm (*e.g.*, diluent control) reached 2000 mm^3, the animals were euthanized compared. As seen in Figure 10, SIN-IL2 treated animals showed a significant anti-tumor response that was at least comparable to the recombinant IL2 protein.

EXAMPLE 4

CHARACTERIZATION OF ALPHAVIRUS REPLICON PARTICLES

To quantitate the number of replicon particles in a preparation, two novel methods are disclosed herein. In the first instance, stable alphavirus packaging cell lines (see for example US 5789245, US 5843723, and WO 99/18226) are provided. The packaging cell lines express each of the alphavirus structural proteins (*e.g.*, capsid, glycoproteins) necessary for production of alphavirus particles, which are not encoded by an alphavirus replicon vector itself. Packaging cell line #15-25 (see above) cells were plated in 6-well dishes to achieve approximately 80-90% confluency at the time of infection. Serial dilutions of a preparation of SIN replicon particles expressing a reporter gene were then diluted serially and used to infect the cells in duplicate, at 37C for 1 hour. Subsequently, the inoculum was removed, the wells overlayed with agarose and the infected cells incubated at 37C. Plaques were visualized 48-72 hours later at which time they could be quantitated directly or after staining with a dye such as neutral red or crystal violet.

In the second instance, nucleic acid based detection of alphavirus replicon particles as a means for quantitation was performed, using the bDNA amplification technique (Wilber,

Immunol Invest 1997, 26:9-13) as one embodiment. Figure 11 shows representative data from an experiment in which the titer of SIN replicon particles expressing HIV-gag antigen was determined. A standard curve was developed initially using serial dilutions of SIN replicon particles expressing GFP reporter, since prior quantitation of this material could be done by direct transfer of expression (TOE) assay and counting of green cells in a fluorescence microscope. As the vector replicon backbone was identical between SIN-GFP and SIN-gag, nucleic acid detection could then be done using an identical nonstructural gene specific probe, since both particle preparations differed only in the expressed heterologous gene.

In addition to quantifying the number of replicon particles in a preparation, it is also advantageous (or necessary) to determine the presence or absence of contaminating replication-competent virus (RCV) in the preparation. Such RCV, if present, would have resulted from RNA recombination during the replicon packaging process. It has long been recognized by those of skill in the art that RCV testing may be performed using standard plaque assay, with or without prior serial passage in naïve cultured cells. In order to increase the level of sensitivity of RCV detection and to detect the multiple recombination events required for the generation of RCV in a "split helper" alphavirus packaging system, a nucleic acid based assay as described herein has been developed (Figure 12). In this assay, a preparation containing replicon particles is first extracted to isolate the nucleic acid substrate (*e.g.*, RNA) present. The nucleic acid substrate is then included in a first PCR reaction mixture comprising a first oligonucleotide complementary to an alphavirus sequence not present in the helper sequence(s) (*e.g.*, nonstructural protein gene-specific, Rep-Fwd in Fig. 12), and a second oligonucleotide complementary to an alphavirus structural protein gene (e.g., DH1 Rev or DH2 Rev, in Fig. 12), wherein the structural protein is either a capsid protein or a non-capsid structural protein (*e.g.*, glycoprotein). A reaction product from this reaction will specifically identify a recombination event between the replicon vector and whichever structural protein gene containing helper the second oligonucleotide was designed complementary to. Thus, for example, if the second oligonucleotide was capsid gene-specific oligonucleotide DH2 Rev, a recombination event between the replicon and the capsid gene-containing helper (*e.g.*, DH2) could be detected by the reaction product. In addition, based on length of the reaction product, multiple recombination events might also be detected at this stage, but one could not necessarily ascertain whether such recombination events included all structural gene elements required for generation of RCV or simply recombination with multiple copies of the same structural protein gene helper (*e.g.*, two copies of capsid from DH2).

Therefore, following amplification, the reaction product(s) from the first reaction mixture is included in a second PCR reaction mixture comprising an oligonucleotide complementary to an alphavirus capsid protein gene (*e.g.*, DH2 Rev) and a oligonucleotide complementary to a non-capsid (*e.g.*, glycoprotein) alphavirus structural protein gene (*e.g.*, DH1 Fwd). For example, if the first reaction resulted in a product that was amplified using replicon and capsid specific oligonucleotides (*e.g.*, Rep-Fwd and DH2 Rev), indicating recombination between replicon RNA and capsid-containing helper RNA, then the ability to synthesize a second reaction product in a second reaction containing the first reaction product as template and oligonucleotides complementary to an alphavirus capsid gene (*e.g.*, DH2 Rev) and non-capsid structural protein gene (*e.g.*, DH1 Fwd), would be indicative of multiple recombination events.

Preferably, two separate first reactions are performed to identify either a capsid gene recombinant (*e.g.*, using Rep-Fwd and DH2 Rev) or a non-capsid structural protein gene recombinant (*e.g.*, using Rep-Fwd and DH1 Rev). Each of these first reactions then would be subjected to a second reaction as described above, allowing for the identification of all possible multiple recombination events that could result in RCV.

Similarly, such an approach may be used to identify packaging of a defective helper RNA into particles within a preparation, as well as co-packaging of replicon and defective helper RNA within particles. For example, the ability to amplify either capsid gene (*e.g.*, DH2 Fwd plus DH2 Rev) or non-capsid structural protein gene (*e.g.*, DH1 Fwd plus DH1 Rev) sequences, without being able to amplify a product resulting from recombination (*e.g.*, Rep-Fwd plus DH1 Rev or DH2 Rev) would be indicative of helper RNA present in packaged particles.

All references including publications, patent applications and patents cited herein are hereby incorporated by reference to the same extent as if each reference was individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The terms "a" and "an" and "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any

and all examples, or exemplary language (e.g. "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on those preferred embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

What is claimed is:

1. A method for purifying alphavirus replicon particles comprising:

a) contacting a preparation containing alphavirus replicon particles with a tentacle ion exchange resin, under conditions and for a time sufficient to bind to said resin;

b) removing the portion of the preparation which is not bound to said ion exchange resin from said ion exchange resin;

c) eluting the bound alphavirus replicon particles from said ion exchange resin; and

d) recovering said replicon particles.

2. The method according to claim 1 wherein said tentacle ion exchange resin is a cationic exchange resin.

3. The method according to claim 1 wherein said tentacle ion exchange resin is an anionic exchange resin.

4. A method for purifying alphavirus replicon particles comprising at least two chromatographic purification steps wherein said purification steps are selected from the group consisting of ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, affinity chromatography.

5. The method according to claim 4 wherein said ion exchange chromatography is performed using a tentacle ion exchange resin.

6. The method according to claim 4 wherein a first purification step is ion exchange chromatography and a second purification step is size exclusion chromatography.

7. An alphavirus replicon particle preparation made according to any one of claims 4-6.

8. An alphavirus replicon particle preparation according to claim 7 wherein said preparation comprises an immunogenic composition capable of expressing an antigen derived from a pathogenic agent.

9. The alphavirus replicon particle preparation according to claim 8 wherein said pathogenic agent is selected from the group consisting of viruses, bacteria, fungi, parasites, and cancerous cells.

10. An alphavirus replicon particle preparation according to claim 7 wherein said alphavirus replicon particle preparation comprises a therapeutic.

11 An alphavirus replicon particle preparation according to claim 10 wherein said alphavirus replicon particle preparation expresses a lymphokine, cytokine, or chemokine.

12. An alphavirus replicon particle preparation according to claim 7 wherein said lymphokine, cytokine or chemokine is selected from the group consisting of IL-2, IL-10, IL-12, gamma interferon, GM-CSF, macrophage inflammatory protein (MIP)3α, MIP3β, and secondary lymphoid tissue chemokine (SLC).

13. A method for stimulating an immune response within a warm-blooded animal, comprising administering to a warm-blooded animal the alphavirus replicon particle preparation of claim 7.

14 The method according to claim 13 wherein said alphavirus replicon particle preparation expresses a lymphokine, cytokine, or chemokine.

15. The method according to claim 14 wherein said lymphokine, cytokine or chemokine is selected from the group consisting of IL-2, IL-10, IL-12, gamma interferon, GM-CSF, macrophage inflammatory protein (MIP)3α, MIP3β, and secondary lymphoid tissue chemokine (SLC).

16. A method of producing alphavirus replicon particles comprising:

a) infecting alphavirus packaging cells with a seed stock of alphavirus replicon particles;

b) incubating the infected packaging cells in a bioreactor, under conditions and for a time sufficient to permit the production of alphavirus replicon particles; and

c) harvesting culture supernatants containing said replicon particles.

17. A method according to claim 16 wherein said bioreactor is an external component bioreactor.

18. A method according to claim 16 wherein said bioreactor is a suspension culture bioreactor.

19. A method according to claim 16 wherein said bioreactor is a hollow fiber bioreactor.

20. A method for producing alphavirus replicon particles comprising:

a) transfecting alphavirus packaging cells with a DNA-based alphavirus replicon or eukaryotic layered vector initiation system;

b) incubating the transfected packaging cells in a bioreactor, under conditions and for a time sufficient to permit the production of alphavirus replicon particles; and

c) harvesting culture supernatants containing said replicon particles.

21. A method for producing alphavirus replicon particles comprising:

a) transfecting alphavirus packaging cells with an alphavirus RNA vector replicon transcribed *in vitro*;

b) incubating the transfected packaging cells in a bioreactor, under conditions and for a time sufficient to permit the production of alphavirus replicon particles; and

c) harvesting culture supernatants containing said replicon particles.

22. A method for detecting multiple recombination events in a population of alphavirus replicon particles comprising:

a) providing a nucleic acid substrate suitable for detecting multiple recombination events, said substrate derived from said population of alphavirus replicon particles;

b) reacting said nucleic acid substrate with at least one first reaction mixture comprising an oligonucleotide complementary to an alphavirus nonstructural protein gene and an oligonucleotide complementary to an alphavirus structural protein gene, wherein said structural protein is either a capsid protein or a non-capsid structural protein, under conditions suitable and for a time sufficient to permit amplification of said nucleic acid substrate to form a first reaction product;

c) reacting said first reaction product with a second reaction mixture comprising a oligonucleotide complementary to an alphavirus capsid protein gene and a oligonucleotide complementary to a non-capsid alphavirus structural protein gene, under conditions suitable and for a time sufficient to permit amplification of said nucleic acid template to form a second reaction product; and

d) determining the presence or absence of said second reaction product.

23. A method for detecting multiple recombination events in a population of alphavirus replicon particles comprising:

a) providing a nucleic acid substrate suitable for detecting multiple recombination events, said substrate derived from said population of alphavirus replicon particles,

b) reacting said nucleic acid substrate with a first reaction mixture comprising an oligonucleotide complementary to an alphavirus nonstructural protein gene and an oligonucleotide complementary to an alphavirus capsid protein gene, under conditions suitable and for a time sufficient to permit amplification of said nucleic acid substrate to form a first reaction product;

c) reacting said first reaction product with a second reaction mixture comprising a oligonucleotide complementary to an alphavirus capsid protein gene and a oligonucleotide complementary to a non-capsid alphavirus structural protein gene, under conditions suitable and

for a time sufficient to permit amplification of said nucleic acid template to form a second reaction product; and

d) determining the presence or absence of said second reaction product.

24. A method for detecting multiple recombination events in a population of alphavirus replicon particles comprising:

a) providing a nucleic acid substrate suitable for detecting multiple recombination events, said substrate derived from said population of alphavirus replicon particles;

b) reacting said nucleic acid substrate with a first reaction mixture comprising an oligonucleotide complementary to an alphavirus nonstructural protein gene and an oligonucleotide complementary to a non-capsid alphavirus structural protein gene, under conditions suitable and for a time sufficient to permit amplification of said nucleic acid substrate to form a first reaction product,

c) reacting said first reaction product with a second reaction mixture comprising a oligonucleotide complementary to an alphavirus capsid protein gene and a oligonucleotide complementary to a non-capsid alphavirus structural protein gene, under conditions suitable and for a time sufficient to permit amplification of said nucleic acid template to form a second reaction product; and

d) determining the presence or absence of said second reaction product

25. A method for quantitating alphavirus replicon vector particles comprising:

a) providing a population of packaging cells;

b) contacting said packaging cells with said alphavirus replicon vector particles under conditions suitable and for a time sufficient for said cells to be infected with said alphavirus replicon vector particles;

c) incubating said infected packaging cells under conditions suitable and for a time sufficient for production of said alphavirus replicon vector particle;

d) enumerating the number of resulting plaques.

26. The method according to claim 25, wherein said packaging cells express all structural proteins necessary for packaging of said alphavirus replicon vector particles.

27. The method according to claim 25, wherein said packaging cells comprises at least one expression cassette expressing an alphavirus capsid protein and at least one alphavirus glycoprotein.

28. The method according to claim 25, wherein said packaging cells express an alphavirus capsid protein and at least one alphavirus glycoprotein from distinct expression cassettes.

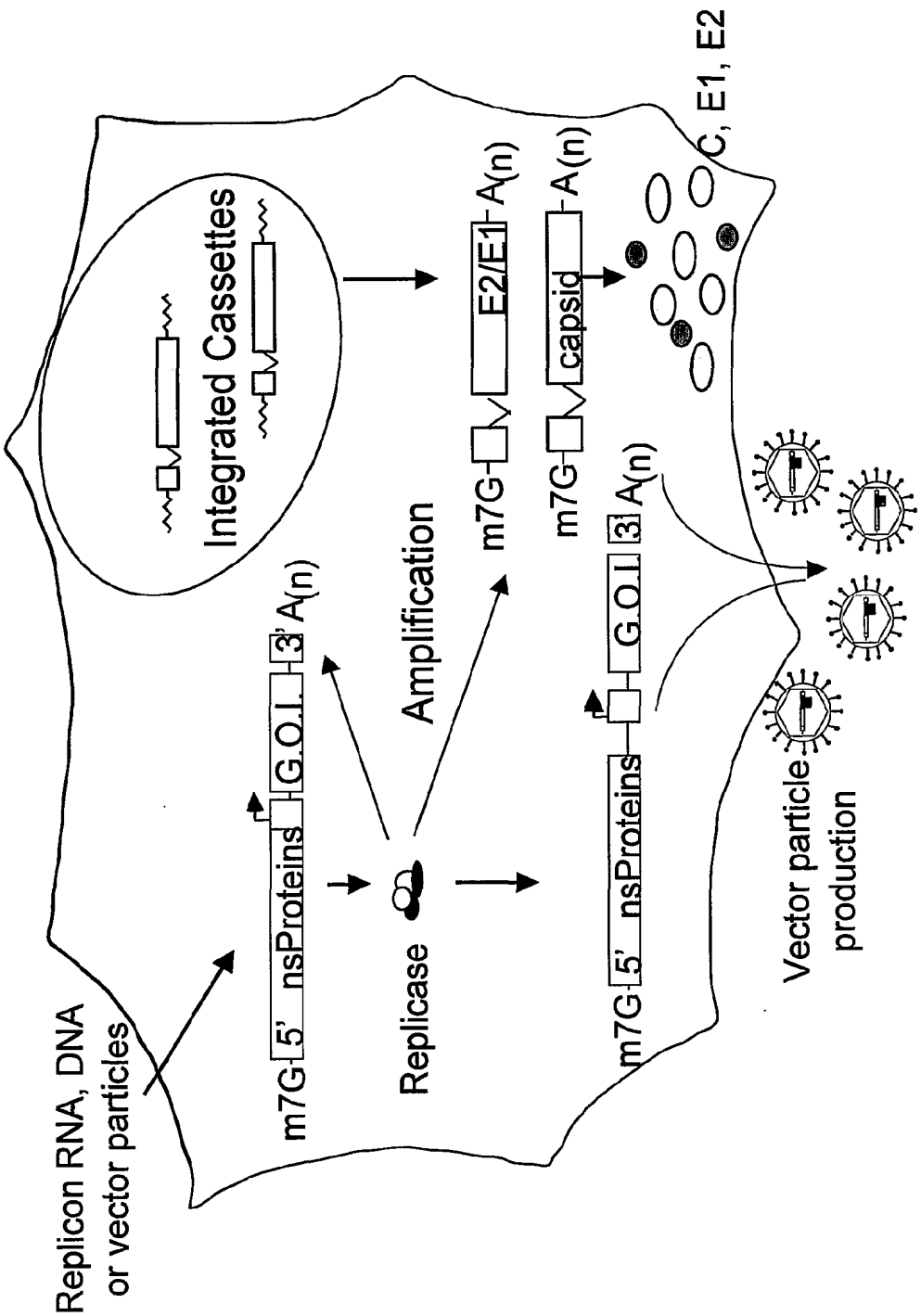

Replicon RNA, DNA
or vector particles
Integrated Cassettes
m7G
5' nsProteins
G.O.I.
3'
A(n)
Replicase
Amplification
m7G
E2/E1
A(n)
m7G
capsid
A(n)
C, E1, E2
m7G
5' nsProteins
G.O.I.
3'
A(n)
Vector particle
production

Figure 1

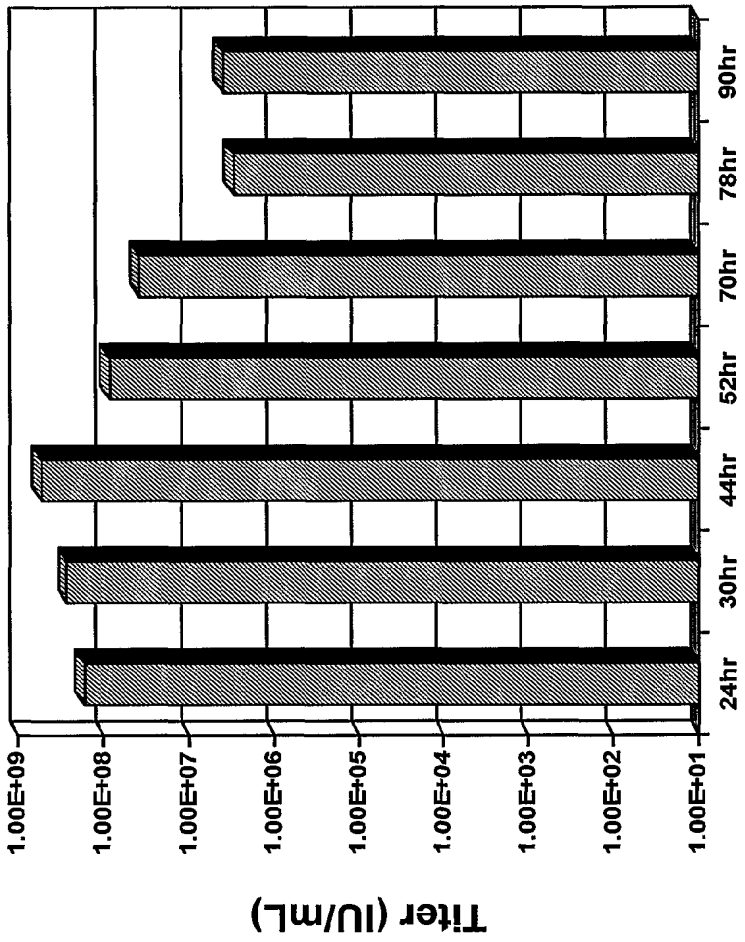

Titer (IU/mL)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
24hr
30hr
44hr
52hr
70hr
78hr
90hr
Time

Figure 2

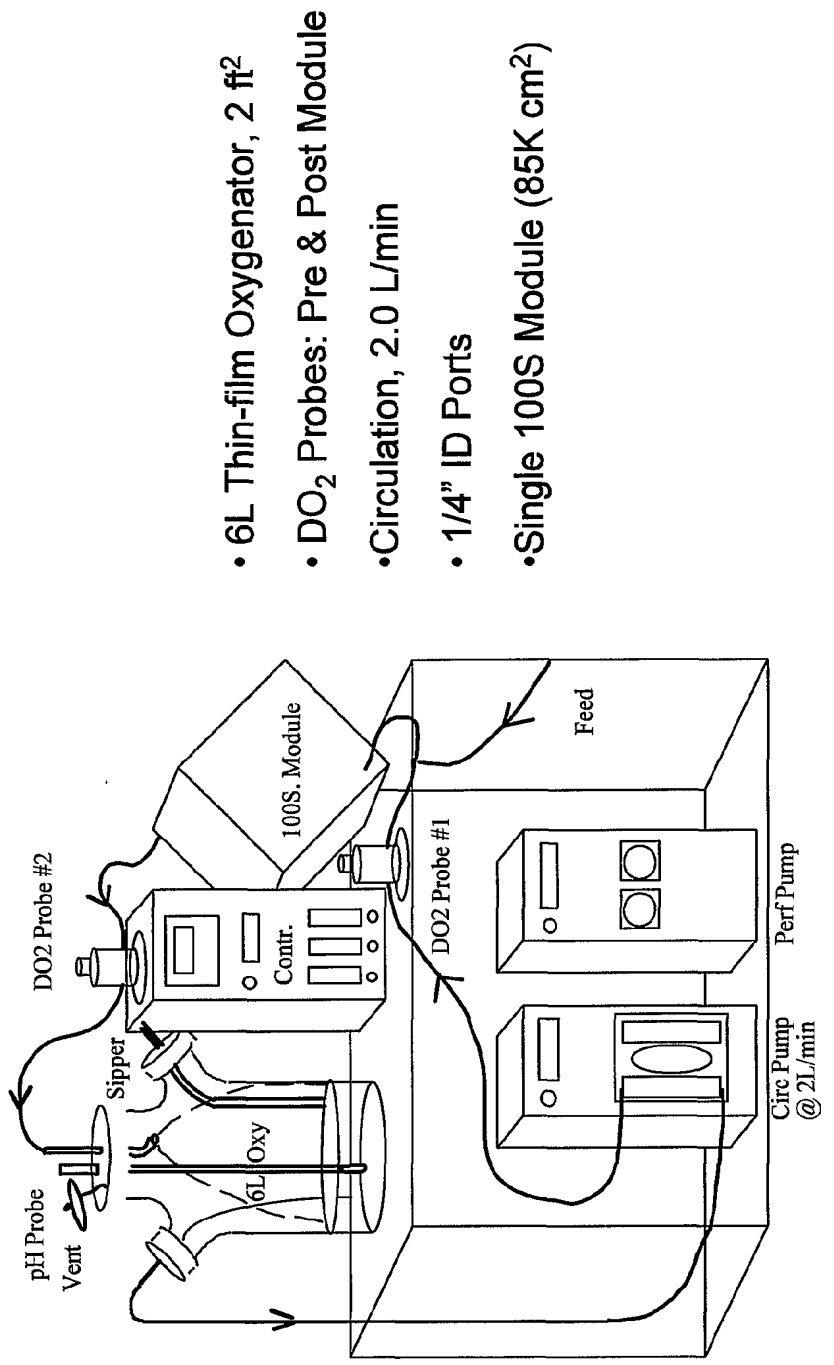

• 6L Thin-film Oxygenator, 2 ft²
• DO₂ Probes: Pre & Post Module
•Circulation, 2.0 L/min
• 1/4" ID Ports
•Single 100S Module (85K cm²)
DO2 Probe #2
pH Probe
Vent
Sipper
6L Oxy
Contr.
100S. Module
DO2 Probe #1
Feed
Perf Pump
Circ Pump @ 2L/min

Figure 3

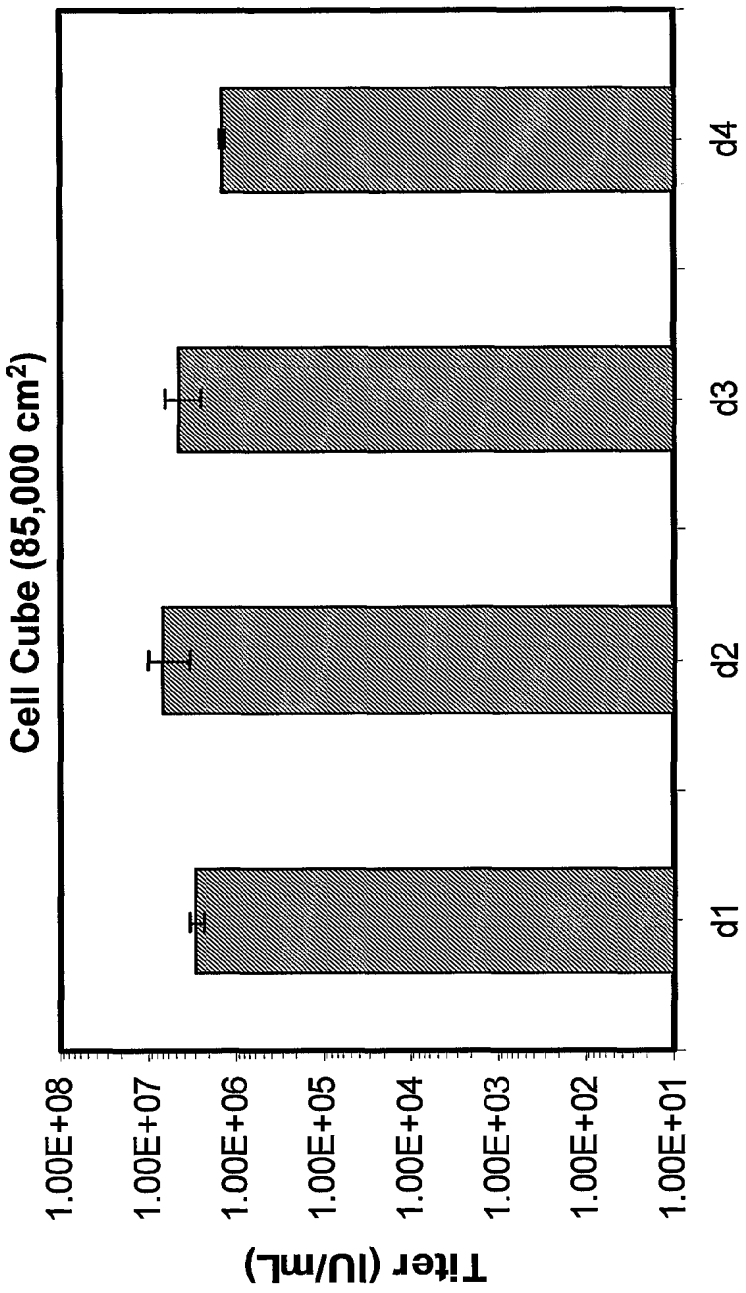

Cell Cube (85,000 cm2)
Titer (IU/mL)
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
d1
d2
d3
d4
Days

Figure 4

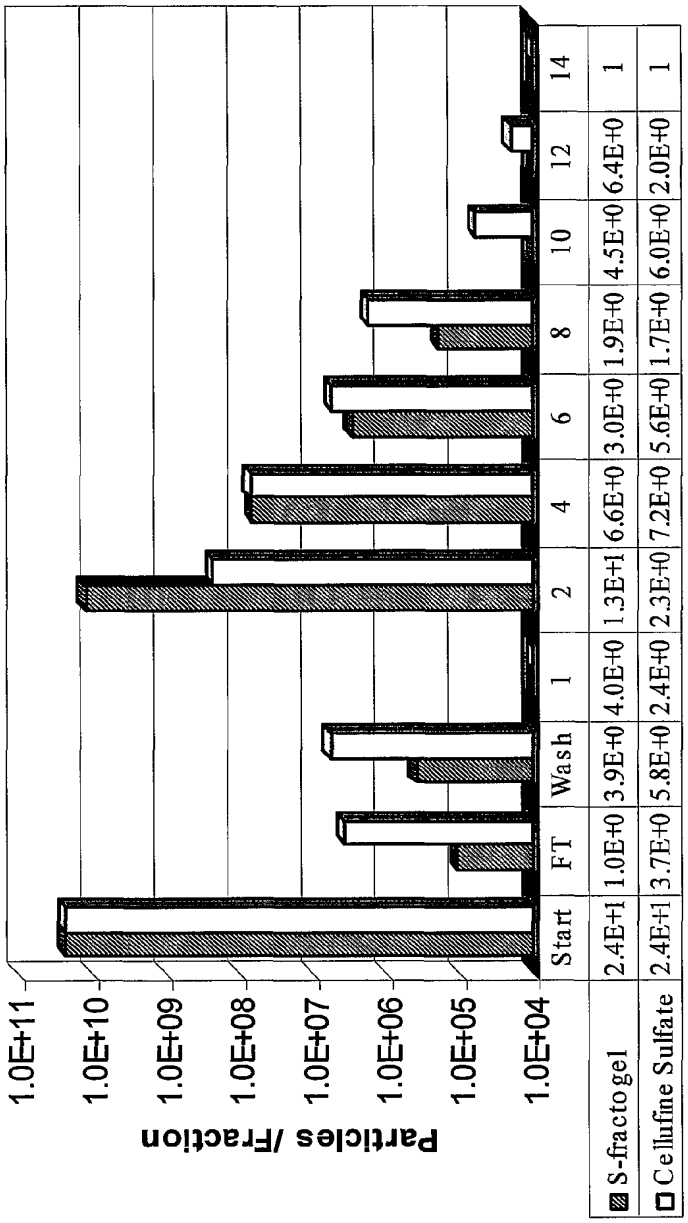

1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
Particles /Fraction
Start FT Wash 1 2 4 6 8 10 12 14
S-fractogel 2.4E+1 1.0E+0 3.9E+0 4.0E+0 1.3E+1 6.6E+0 3.0E+0 1.9E+0 4.5E+0 6.4E+0 1
Cellufine Sulfate 2.4E+1 3.7E+0 5.8E+0 2.4E+0 2.3E+0 7.2E+0 5.6E+0 1.7E+0 6.0E+0 2.0E+0 1
Fractions

Figure 5

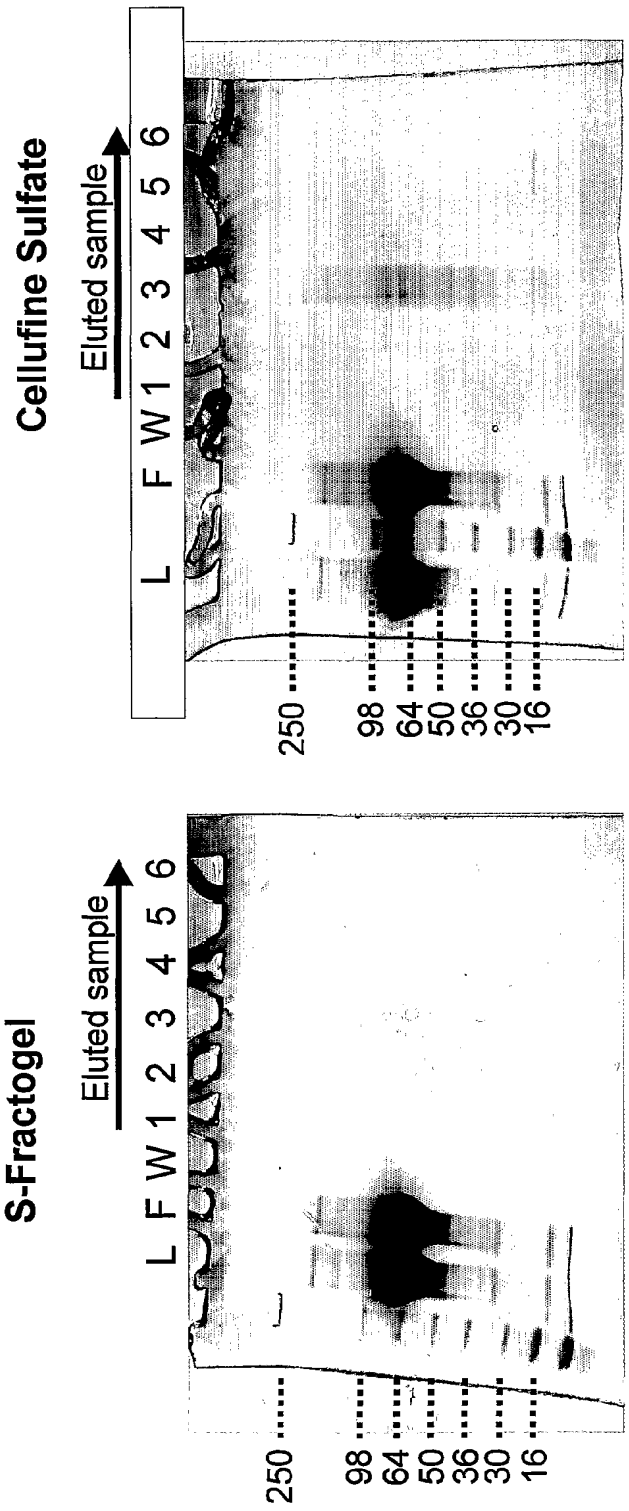

Cellufine Sulfate
Eluted sample
L F W 1 2 3 4 5 6
250
98
64
50
36
30
16
S-Fractogel
Eluted sample
L F W 1 2 3 4 5 6
250
98
64
50
36
30
16

Figure 6

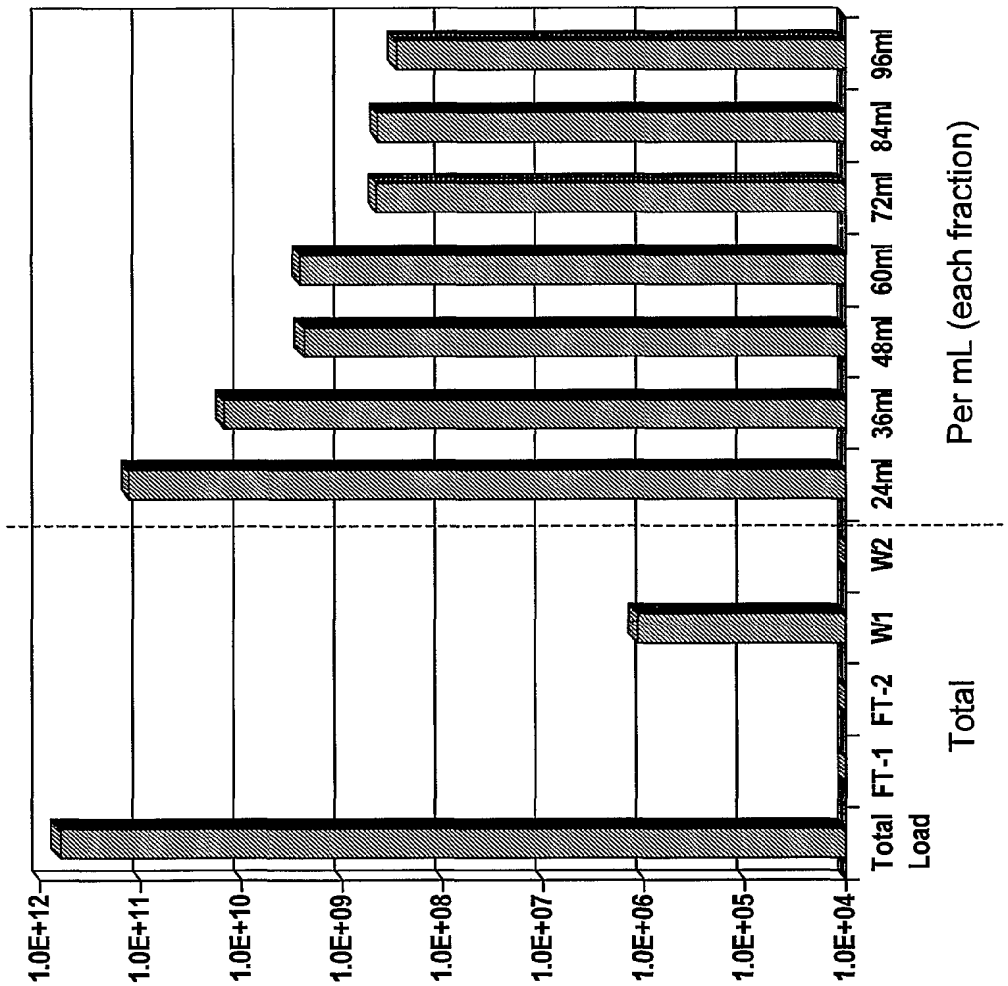

1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
Total Load
FT-1
FT-2
W1
W2
24ml
36ml
48ml
60ml
72ml
84ml
96ml
Per mL (each fraction)
Total

Figure 7

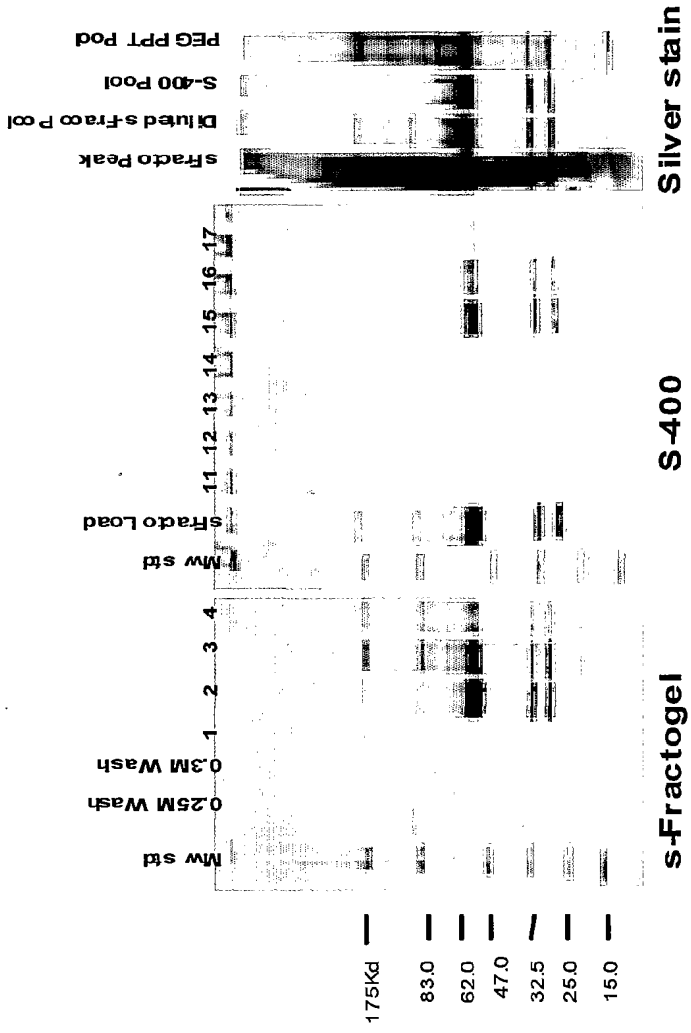

Silver stain
S-400
s-Fractogel
Mw std
0.25M Wash
0.3M Wash
1
2
3
4
Mw std
sFradto Load
11
12
13
14
15
16
17
sFradto Peak
Diluted s-Frac Pool
S-400 Pool
PEG PPT Pod
175Kd
83.0
62.0
47.0
32.5
25.0
15.0

Figure 8

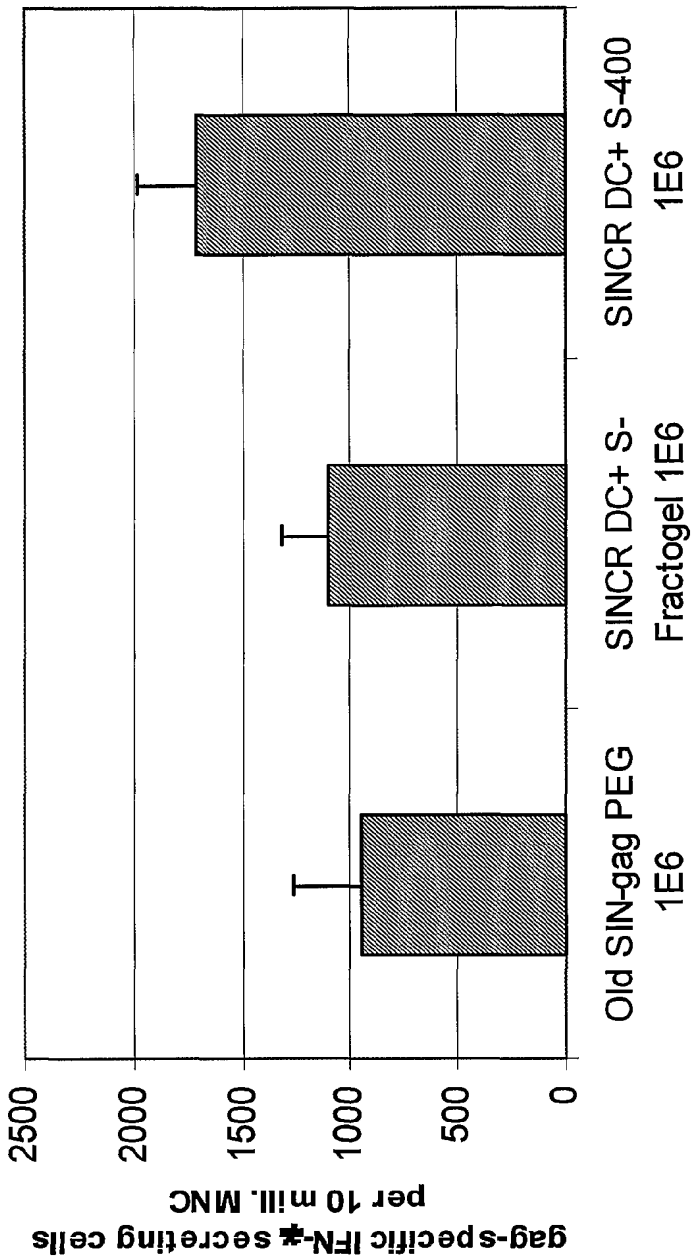

gag-specific IFN-γ secreting cells
per 10 mill. MNC
2500
2000
1500
1000
500
0
Old SIN-gag PEG 1E6
SINCR DC+ S-Fractogel 1E6
SINCR DC+ S-400 1E6

Figure 9

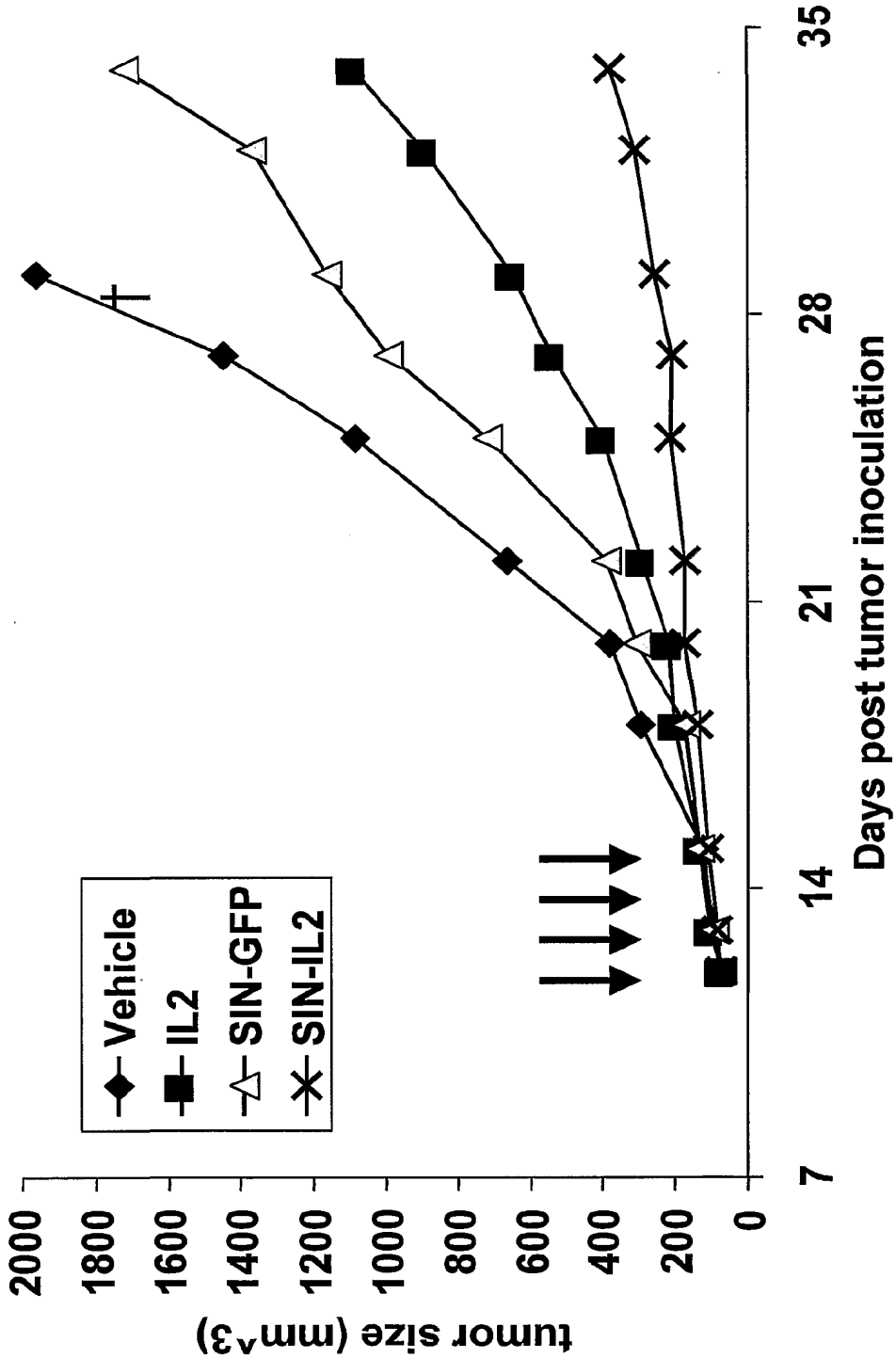

tumor size (mm^3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
7
14
21
28
35
Days post tumor inoculation
Vehicle
IL2
SIN-GFP
SIN-IL2

Figure 10

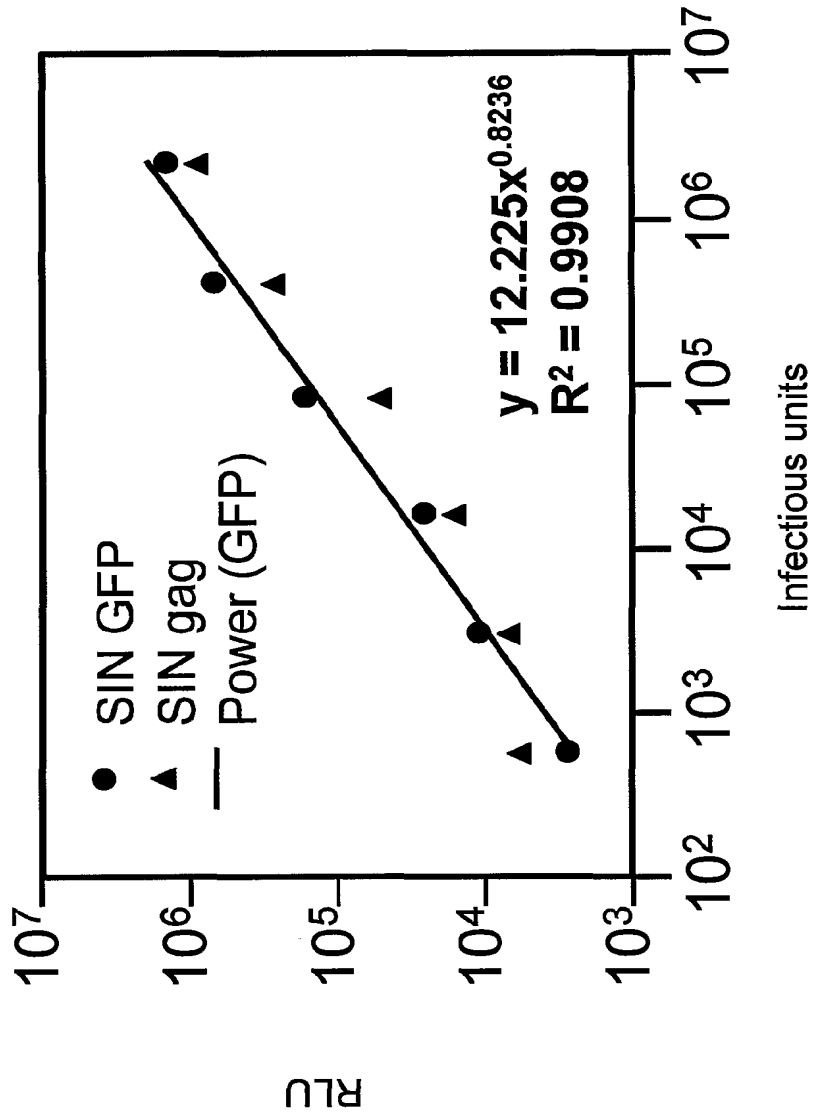

RLU
Infectious units
SIN GFP
SIN gag
Power (GFP)
y = 12.225x^0.8236
R² = 0.9908
10^7
10^6
10^5
10^4
10^3
10^2
10^3
10^4
10^5
10^6
10^7

Figure 11

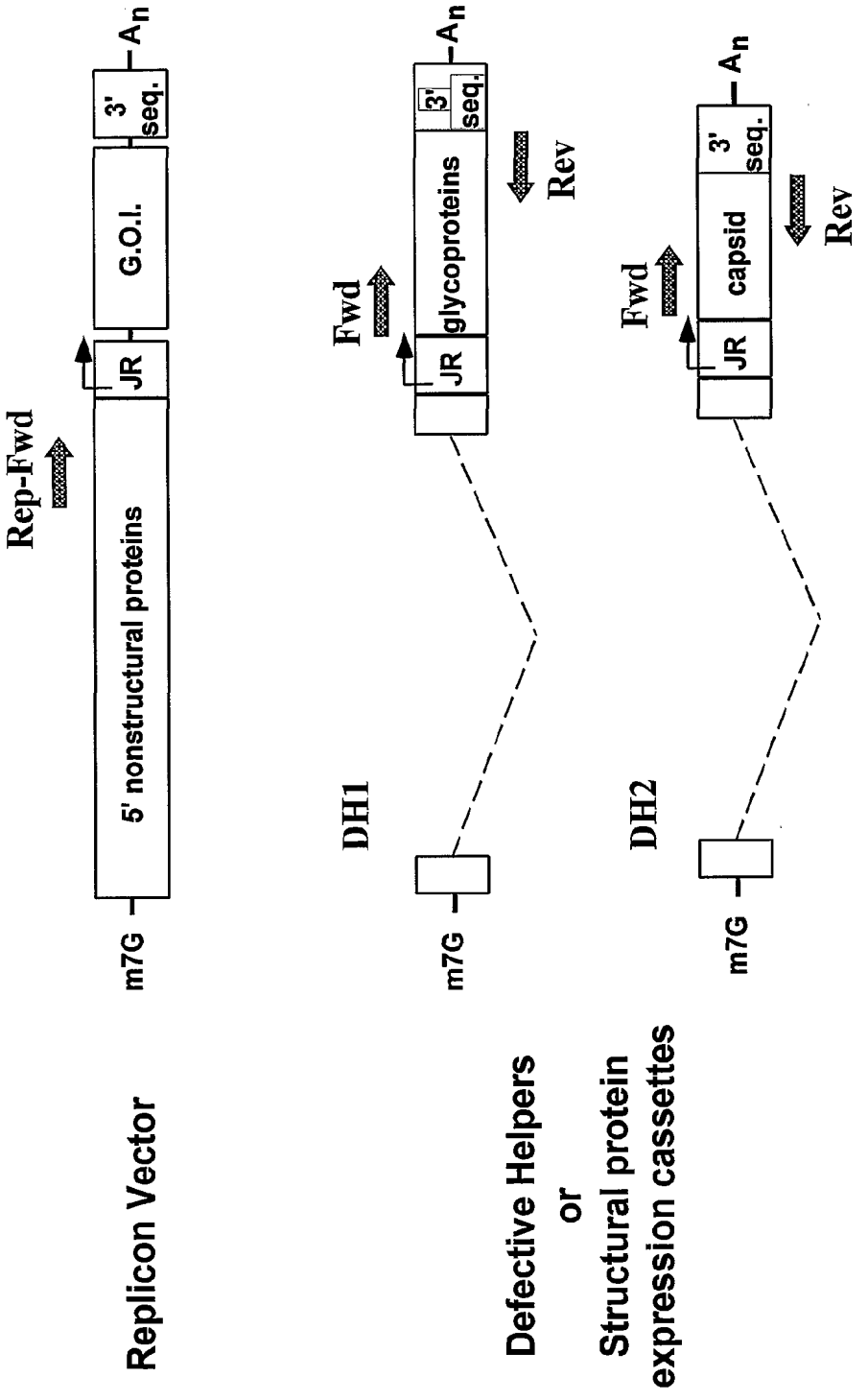

Rep-Fwd
5' nonstructural proteins
JR
G.O.I.
3' seq.
An
m7G
Replicon Vector
Fwd
JR
glycoproteins
3' seq.
Rev
DH1
m7G
Fwd
JR
capsid
3' seq.
Rev
DH2
m7G
Defective Helpers or Structural protein expression cassettes

Figure 12

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(54) **Title**: ALUMINIUM COMPOUNDS FOR USE IN THERAPEUTICS AND VACCINES

(57) **Abstract**: The invention relates to means and methods for preparing aqueous composition comprising aluminium and a protein. The invention further relates to aqueous compositions comprising a protein and an aluminium-salt, said composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition.

Title: **Aluminium compounds for use in therapeutics and vaccines**

The invention relates to the fields of pharmaceuticals and vaccines. More in particular the invention relates to the field of compounds and compositions that are being co-administered with the medicament and/or antigen.

Aluminium compounds (herein also referred to as "aluminium"), including aluminium phosphate (AlPO4), aluminium hydroxide (Al(OH)3), and other aluminium precipitated vaccines are currently the most commonly used adjuvants with human and veterinary vaccines. The adjuvants are often referred to as "alum" in the literature.

Aluminium adjuvants have been used in practical vaccination for more than half a century. They induce early, high-titre, long-lasting protective immunity. Billions of doses of aluminium-adjuvanted vaccines have been administered over the years. Their safety and efficacy have made them the most popular adjuvants in vaccines to date. In general, aluminium adjuvants are regarded as safe when used in accordance with current vaccination schedules.

In human vaccinations, of old, aluminium adjuvants have been used in tetanus, diphtheria, pertussis and poliomyelitis vaccines as part of standard child vaccination programmes. Aluminium adjuvants have also been introduced into hepatitis A and hepatitis B virus vaccines and Japanese encephalitis virus (also referred herein as "JEV") vaccines. Other aluminium-adsorbed vaccines against, for example, anthrax, are available for special risk groups. In veterinary medicine aluminium adjuvants have been used in a large number of vaccine formulations against viral and bacterial diseases, and in attempts to make anti-parasite vaccines.

Adjuvants typically serve to bring the antigen, the substance that stimulates the specific protective immune response, into contact with the immune system and influence the type of immunity produced, as well as the quality of the immune response (magnitude or duration); Adjuvants can also decrease the toxicity of certain antigens; and provide solubility to some vaccines components. Studies have shown that many aluminium-containing

vaccines cause higher and more prolonged antibody responses than comparable vaccines without the adjuvant. The benefit of adjuvants has usually been observed during the initial immunization series rather than with booster doses.

There are three general types of aluminium-containing adjuvants:

Aluminium hydroxide, Aluminium phosphate and Potassium aluminium sulphate (collectively often referred to as "Alum")

The effectiveness of each salt as an adjuvant depends on the characteristics of the specific vaccine and how the manufacturer prepares the vaccine. To work as an adjuvant, the antigen is typically adsorbed to the aluminium; that is, it is clumped with the aluminium salt to keep the antigen at the site of injection.

Not all vaccines contain aluminium salts. Sometimes an adjuvant may not have been needed or another adjuvant was selected. Examples of commercial vaccines that do not contain aluminium salts are inactivated Polio Virus (IPV) vaccine, measles, mumps and rubella vaccine (MMR), varicella vaccine, Meningococcal conjugate (MCV4) vaccine, and influenza vaccines. That the commercial vaccines do not contain aluminium salts does typically not mean that an aluminium salt would not work. It just means that for some reason another adjuvant was selected.

Examples of US licensed vaccines for children that contain aluminium adjuvants are: DTP (diphtheria-tetanus-pertussis vaccine); DTaP (diphtheria-tetanus-acellular pertussis vaccine); some but not all Hib (Haemophilus influenzae type b) conjugate vaccines; Pneumococcal conjugate vaccine; Hepatitis B vaccines; Hepatitis A vaccines; Human Papillomavirus vaccine; Anthrax vaccine; and Rabies vaccine.

Aluminium is a very abundant element in our environment. It is in many foods we eat, many personal hygiene products we apply to our skin (deodorants, for example), and many medicines we ingest. Various government agencies establish guidelines for exposure to potentially toxic substances.

These guidelines are called “minimal risk levels” —the maximum amount that one can be exposed to over time-usually on a daily basis-without expected harm.

The US Agency for Toxic Substances and Disease Registry (ATSDR) estimated these levels for infants taking into account the amount of aluminium (e.g. in form of a salt) a child would eat as well as receive by injection of vaccines. The body burden of aluminium from both sources is below the minimal risk level except transiently following vaccinations; since 50-70% of injected aluminium is excreted within 24 hours, this is believed to have no negative effect.

Aluminium hydroxide and aluminium phosphate adjuvants are generally prepared by exposing aqueous solutions of aluminium ions, to usually slightly alkaline conditions in a well-defined and controlled chemical environment. Various soluble aluminium salts can be used for the production of aluminium hydroxide. Anions present at the time of precipitation may coprecipitate (for review see, Lindblad, EB (2004) Immunol. and Cell Biol. Vol 82: 497-505).

Aluminium salt is also used in the manufacture and composition of medicaments. For instance, factor VIII is purified from plasma cryoprecipitate. The precipitate is solubilised, absorbed on aluminium hydroxide and then treated to inactivate lipid enveloped viruses. After several other processing steps the concentrate is used to treat hemophilia A patients (Burnouf T, (1991) Vox Sang. Vol 60: pp 8-15).

In the present invention it has been shown that stability of a biological in a composition that also comprises an aluminium salt is not always the same. The present invention, for instance, shows that the stability of a protein component (e.g. as such or within a complex such as e.g. a virus or other pathogen) in the context of an aqueous composition that also comprises aluminium salt is dependent on the content of heavy metals. To estimate a priori whether the protein will be stable in this composition, the present invention provides that it is necessary to determine the residual heavy metal

content in the composition (otherwise the aqueous composition comprising a protein is at risk of being degraded over time in particular the invention provides that this risk is considerable when the residual heavy metal content is above 350 ppb (i.e. about 350ng per ml) in said aqueous composition). Further, the invention also revealed that this residual heavy metal content cannot easily removed from the aluminium compound. To this end the invention provides a method for preparing an aqueous composition comprising aluminium and a protein said method comprising

- combining an aluminium-salt, said protein and water to produce said aqueous composition and
- determining the level of a heavy metal in the aqueous composition and/or the aluminium-salt. Compositions comprising less than 350 ppb heavy metal based on the weight of the aqueous composition, can be stored in a liquid phase at a temperature of between 0 and 30 degrees Celsius, for at least 1 month, such as e.g. 20 months at 2-8°C. The protein component in said composition is stable for at least 1 month in said liquid phase. Compositions comprising more than 350 ppb heavy metal based on the weight of the aqueous composition cannot be stored for a prolonged period under such conditions as the protein component in said composition changes in at least one aspect over the indicated time period. One millilitre or one gram of aqueous composition thus preferably contains no more than 350 nanogram heavy metal. The aqueous composition preferably comprises between 0,1 mg/ml and 2,5 mg/ml aluminium. The average dose of aluminium per administration is preferably not more than 1,25 milligram (mgram). In a particularly preferred embodiment the dose of aluminium per administration is not more than 0,25 mgram aluminium. A dose typically comprises between 0,5 and 1 ml of the aqueous composition.

In a further aspect of the present invention it has been shown that stability of a biological in a composition that comprises an aluminium salt and a reactive compound is not always the same. The present invention, for

instance, shows that the stability of a protein component (e.g. as such or within a complex such as e.g. a virus or other pathogen) in the context of an aqueous composition that also comprises aluminium salt and a reactive compound such as e.g. a sulphite is critically dependent on the content of heavy metals. To estimate a priori whether the protein component (such as e.g. protein component within a complex such as e.g. a virus particle; herein also referred to simply as protein) will be stable in this composition, it is necessary to determine the heavy metal content in the composition. To this end the invention provides a method for preparing an aqueous composition comprising aluminium and a protein said method comprising

- combining an aluminium-salt, said protein and water to produce said aqueous composition and

- determining the level of a heavy metal in the aqueous composition and/or the aluminium-salt. Compositions comprising less than 350 ppb heavy metal based on the weight of the aqueous composition, can be stored in a liquid phase at a temperature of between 0 and 30 degrees Celsius, for at least 1 month, such as e.g. 20 months at 2-8°C. The protein component in said composition is stable for at least 1 month in said liquid phase, such as e.g. 20 months at 2-8°C. Compositions comprising more than 350 ppb heavy metal based on the weight of the aqueous composition cannot be stored for a prolonged period under such conditions as the protein component in said composition changes in at least one aspect over the indicated time period. One millilitre or one gram of aqueous composition thus preferably contains no more than 350 nanogram heavy metal. The aqueous composition preferably comprises between 0,1 mg/ml (milligram per millilitre) and 2,5 mg/ml aluminium. The average dose of aluminium per administration is preferably not more than 1,25 milligram (mgram). In a particularly preferred embodiment the dose of aluminium per administration is not more than 0,25 mgram aluminium. A dose typically comprises between 0,5 and 1 ml of the aqueous composition. An aqueous composition comprising a protein, an

aluminium-salt, and optionally a reactive compound, said composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition is herein also referred to as "an aqueous composition comprising a protein according to the invention" or "a composition comprising a protein according to the invention".

The aluminium content of the aqueous composition is usually around 0,1 mg/ml to 2,5 mg/ml, with the average vaccine comprising approximately 0,5 to 1,5 mg/ml of the aluminium adjuvant. To this end the invention provides an aqueous composition, an aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein, comprising between 5 mcgram/ml and 50 mg/ml aluminium and comprising no more than 700 ppm of a heavy metal when compared to the aluminium content (gram/gram). In this context 700 ppm of a heavy metal equals 700 mcgram heavy metal per gram of aluminium.

In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein, comprises between 5 mcgram/ml and 50 mg/ml aluminium and comprises no more than 450 ppm of a heavy metal when compared to the aluminium content (gram/gram). In this context 450 ppm of a heavy metal equals 450 mcgram heavy metal per gram of aluminium.

In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein, comprises between 5 mcgram/ml and 50 mg/ml aluminium and comprises no more than 700 ppm of Fe when compared to the aluminium content (gram/gram). Preferably the Fe content is less than 420 ppm Fe when compared to the aluminium content. Preferably the Fe content is less than 350 ppm, preferably less than 100 and more preferably less than 50 ppm when compared to the aluminium content (1 ppm = 1 mcgram/gram Al). In a particularly preferred embodiment the Fe content of the aqueous composition, the aqueous pharmaceutical or vaccine is less than 420 ppm when

compared to the aluminium content. In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine comprises more than 10 ppm Fe when compared to the aluminium content.

In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein, comprises between 5 mcgram/ml and 50 mg/ml aluminium and comprises no more than 35 ppm of Ni when compared to the aluminium content (gram/gram). Preferably the Ni content is less than 18 ppm Ni when compared to the aluminium content. Preferably the Ni content is less than 9 ppm, preferably less than 3 and more preferably less than 1 ppm when compared to the aluminium content (1 ppm = 1 mcgram/gram Al). In a particularly preferred embodiment the Ni content of the aqueous composition, the aqueous pharmaceutical or vaccine is less than 18 ppm when compared to the aluminium content. In a preferred embodiment the aqueous composition, an aqueous pharmaceutical or vaccine composition comprises at least 200 ppb Ni when compared to the aluminium content.

In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein, comprises between 5 mcgram/ml and 50 mg/ml aluminium and comprises no more than 5 ppm of Cu when compared to the aluminium content (gram/gram). Preferably the Cu content is less than 2,5 ppm Cu when compared to the aluminium content. Preferably the Cu content is less than 1 ppm, preferably less than 0,5 and more preferably less than 0,25 ppm when compared to the aluminium content (1 ppm = 1 mcgram/gram Al). In a particularly preferred embodiment the Cu content of the aqueous composition, the aqueous pharmaceutical or vaccine is less than 2,5 ppm when compared to the aluminium content. In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine composition comprises at least 50 ppb Cu when compared to the aluminium content.

In a particularly preferred embodiment the Fe content of the aqueous composition, the aqueous pharmaceutical or vaccine is less than 420 ppm when compared to the aluminium content, the Ni content of the aqueous composition, the aqueous pharmaceutical or vaccine is less than 18 ppm when compared to the aluminium content and the Cu content of the aqueous composition, the aqueous pharmaceutical or vaccine is less than 2,5 ppm when compared to the aluminium content. In a preferred embodiment the aqueous composition, the aqueous pharmaceutical or vaccine comprises more than 10 ppm Fe when compared to the aluminium content, at least 200 ppb Ni when compared to the aluminium content, and at least 50 ppb Cu when compared to the aluminium content.

In a method for preparing an aqueous composition, an aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein according to the invention, or a method for preparing or selecting an aluminium salt according to the invention, or a method for preparing a clinical grade aluminium salt precipitate according to the invention, it is preferred that the heavy metal content in relation to the aluminium content is as indicted herein above. The invention therefore provides a method for preparing an aqueous composition comprising aluminium, a reactive compound and a protein said method comprising

- preparing or selecting an aluminium-salt that is able to provide an aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition and

- combining said aluminium salt, said reactive compound, said protein and water to produce said aqueous composition,

whereby the aqueous composition comprises between 5 mcgram/ml and 50 mg/ml aluminium, preferably between 0,1 mg/ml and 2,5 mg/ml; and no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content; wherein said aqueous composition comprises no more than 420 ppm Fe, preferably no more than 100

ppm, when compared to the aluminium content; no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content; and/or no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content. In a preferred embodiment, said aqueous composition comprises no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content; no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content; no more than 18 ppm, preferably no more than 3 ppm of Ni when compared to the aluminium content; and no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content.

It has been observed that the aluminium component is an important source for the heavy metal in the aqueous composition. Thus one way to control t he amount of heavy metal in the aqueous composition is to control the amount of heavy metal in the aluminium source used to generate the aqueous composition. The invention therefore further provides a method for preparing an aqueous composition comprising aluminium and a protein said method comprising

- preparing or selecting an aluminium-salt solution (such as e.g. 10mg/ml aluminium hydroxide liquid (such as e.g. alhydrogel® 2% from Brenntag Biosector, catalogue number 843261) that in the final protein formulation comprises no more than 350ppb of heavy metal based on the weight of the aqueous composition (e.g. for the alhydrogel® 2% and final amount of 0,25 mgram aluminium hydroxide in the aqueous composition of 0.5ml (= dose), the selected or prepared aluminium-salt solution should not contain more than 7 microgram heavy metal pro millilitre of the alhydrogel® 2% solution, about 7ppm heavy metal content), and
- combining said aluminium-salt solution, said protein, water and optionally a reactive compound to produce said aqueous composition (with no more than 350ppb heavy metal in the aqueous composition).

For example, the aluminium-salt solution, e.g. the aluminium hydroxide liquid (used as a component to be mixed to result in the final aqueous composition) should not have a heavy metal content higher than 7ppm (given as an example herein where the 10 mg/ml aluminium hydroxide liquid will be diluted to 0,5 mg/ml to result in about 350ppb heavy metal content in relation to the aqueous composition, assuming 1ppm = about 1mg/ml) on the basis of weight of the aluminium hydroxide liquid. The limit of acceptable heavy metal content may also be expressed in relation to the weight of the aluminium-salt such as the aluminium hydroxide in solution (referred to also as "starting aluminium compound"). The acceptable heavy metal content in this example then may not exceed 7 microgram of heavy metal for each gram of aluminium hydroxide solution (i.e. about 7ppm), i.e. the alhydrogel® 2% solution. As indicated herein above the aqueous composition comprising the protein preferably (such as e.g. if used as a vaccine) comprises between 0,1 to 2,5 mg/ml of the aluminium compound. The concentration of heavy metal in the aqueous composition however should according to the invention not exceed 350ppb, i.e. about 350ng per ml of the final composition and thus the selection or preparation of the starting aluminium compound has to be made accordingly. In order to further illustrate the selection of an appropriate starting aluminium compound solution (e.g. in the form of a concentrated solution (see above alhydrogel® 2%=10mg/ml), it is shown that an aluminium compound solution of 10mg/ml aluminium hydroxide having about 7 ppm heavy metal impurities corresponds to a concentration of heavy metal in the protein composition when the aluminium concentration is about 0.1mg/ml of about 70 nanogram/ml or 70 ppb (so well below the 350ppb provided as the limit of the heavy metal content as taught by the invention). A concentration of 2,5 mg/ml of the aluminium hydroxide in the final aqueous composition starting with an aluminium solution of 10mg/ml aluminium hydroxide having about 7 ppm heavy metal impurities will result in a heavy metal content in the aqueous protein composition that corresponds to a concentration of heavy

metal of about 1,75 microgram/ml or about 1,750ppm (so well above the 350ppb provided as the limit of heavy metal content as taught by the invention). Thus, the aluminium solution of 10mg/ml aluminium hydroxide in this case (final aluminium hydroxide content is 2.5 mg/ml) should then have not more than 1.4ppm heavy metal impurities.

A method of preparing an aqueous composition comprising a protein preferably further comprises packaging aliquots of said aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition in separate air-tight storage containers. The protein in the air-tight storage containers is stable and can be stored for at least three months, such as e.g. 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 months, preferably 20 or 24 months, more preferably 20 months at a temperature of between 2 to 8°C degrees Celsius.

Without being bound to theory, antigen degradation in aqueous compositions, such as immunogenic composition, comprising heavy metal ions present in an aluminium salt, such as aluminium hydroxide, might be explained with an underlying degradation pathway assuming free-radicals such as e.g. free-radicals of sulphite. Formation of free radicals can be catalysed by heavy metal ions present in, for instance, aluminium hydroxide and this effect (in case of a certain amount of heavy metal as indicated according to the invention) could be the underlying root cause mechanism for stability issues as identified as part of the inventive contribution. The experimental part of this application shows in great detail the evidence of this root cause for the Japanese encephalitis vaccine (also referred to as “JEV”) and makes a similar showing for a simple aluminium adjuvanted polypeptide composition that comprises a reactive compound such as sulphite. Thus, it is evident that similar reaction may occur also in other aqueous composition comprising aluminium (with high, e.g. higher than 350ppb based on the weight of the composition, heavy metal content), protein and possibly a reactive component such as sulphite and/or other radical forming particles. Heavy

metal-catalysed oxidation is a degradation pathway resulting in covalent modification of proteins. The modified physicochemical properties of the oxidized/modified protein or antigen may result in loss of biological activity (Li et al., 1995; Mayo et al., 2003; Stadtman, 1990).

The following reaction schemes (as possibly occurring in the JEV product as described in the experimental part) are indicative for compositions of the invention comprising in addition to the protein and the heavy metal also sulphite and/or another reactive compound such as e.g. sulphite and/or formaldehyde.

Sodium-metabisulfit ($Na_2S_2O_5$) in solution dissolutes into bisulphite ($S_2O_5^{2-}$), in an alkaline solution the bisulphite equilibrium is towards sulphite (SO_3^{2-}) and in an acidic solution towards H_2SO_3/SO_2. After dissolution of $Na_2S_2O_5$ in water, it is hydrolysed into $NaHSO_3$ as follows

$Na_2S_2O_5 \Leftrightarrow 2\ NaHSO_3$

At neutral pH we can assume the following equilibrium:

$HSO_3^- \Leftrightarrow H^+ + SO_3^{2-}$ pKa=7.2

This means that at pH 7 the equilibrium is shifted towards HSO3- and at more basic conditions (e.g. pH 8) towards SO_3^{2-}.

Formaldehyde forms a bisulphite adduct during neutralization according to the following equation:

$CH_2O + HSO_3^- \rightarrow \quad CH_2(OH)(SO_3)^-$

After bisulphate is used up, the reaction proceeds until equilibrium is reached as follows:

$CH_2O + SO_3^{2-} + H_2O \rightarrow CH_2(OH)(SO_3)^- + OH^-$

Formaldehyde and sulphite react with each other, however, formaldehyde and sulphite have been found to be still present in equilibrium in the JEV vaccine and can be detected in the following range (n=49):

Release Results in DS	**Free Sulphite**	**Free Formaldehyde**
Average (ppm) Average (mM)	113.9 1.41	41.9 1.36
Standard deviation (ppm)	24	14.3
Min (ppm)	66	10.6
Max (ppm)	174	78.7

According to the literature (Ranguelova et al., 2010), transition metal ions catalyse the auto-oxidation of (bi)sulphite via sulphur trioxide anion radical ($\cdot SO_3^-$) formation:

$M^{n+} + SO_3^{2-} \rightarrow M^{(n-1)+} + \cdot SO_3^-$

where M may be copper (Cu^{2+}), iron (Fe^{3+}), oxivanadium (VO^{2+}), manganese (Mn^{2+}), Nickel (Ni^{2+}) or chromate anion (CrO_4^{2-}) (Alipazaga et al. 2004; Berglund et al. 1993; Brandt and Elding 1998; Lima et al. 2002; Shi 1994).

It was shown that such sulphite radicals are highly reactive and can oxidize various substances, such as ascorbate, Hydroquinone and Histidine (Huie at al., 1985). A review of free radical chemistry of sulphite was published by Neta & Huie, 1985. The authors also show that radical formation can be also catalysed by photoionization of sulphite as follows:

$SO_3^{2-} + h\nu \rightarrow \cdot SO_3^- + e^-$

Radical formation catalysed by light might also explain differences observed in potency and ELISA results of unlabeled naked syringes (used for release testing and reference purposes) and fully packaged final vaccine lot samples for the JEV product. Fully packed samples are completely protected

from light, whereas unlabeled syringes might be exposed to light during storage and handling.

An important reaction of the sulphite radical in auto-oxidation systems is with molecular oxygen to form a peroxyl radical which is much more reactive:

$\cdot SO_3^- + O_2 \rightarrow \cdot SO_5^-$

The solubility of O_2 in water at 0°C and 20°C is 0.4mM and 0.25mM, respectively. Assuming that O_2 solubility in a composition of the invention is in a similar range, a considerable amount of oxygen is present to form the peroxyl radical. This radical is a much stronger oxidant compared to $\cdot SO_3^-$ and can oxidize certain substrates which are not attached by $\cdot SO_3^-$ at all and which, in fact, can form radicals that oxidize sulphite ions. In such cases, when the redox potential of the substrate is intermediate between those of $\cdot SO_3^-$ and $\cdot SO_5^-$, a reaction chain is likely to develop in presence of O2 following the general pattern:

$\cdot SO_3^- + O_2 \rightarrow \cdot SO_5^-$

$\cdot SO_5^- + X \rightarrow SO_5^{2-} + \cdot X^+$

$\cdot X^+ + SO_3^{2-} \rightarrow X + \cdot SO_3^-$

The intermediacy of a substrate X may enhance the chain process of sulphite oxidation by oxygen. The one-electron reduction of $\cdot SO_5^-$ yields HSO_5^- (peroxymonosulfate), a very strong oxidant that is capable to oxidize many organic compounds (Lambeth et al., 1973; Ito & Kawanashi, 1991). Peroxymonosulfate is also a precursor sulphate anion radical $\cdot SO_4^-$.

$\cdot SO_5^- + HSO_3^- \rightarrow \cdot SO_4^-. + HSO_4^-$

The $\cdot SO_4^-$.radical is a very strong oxidant, nearly as strong as the hydroxyl radical ($\cdot OH$), and is very likely to oxidize other biomolecules by one-electron oxidation.

In a preferred embodiment, the composition comprising a protein according to the invention is a therapeutic composition or an immunogenic composition, such as a vaccine. Therapeutic compositions are administered to individuals, such as a human or an animal. In particular for such

compositions, it is important that the protein within the composition still has its therapeutic effect at the time it is administered to said individual. Degradation of the protein or changes to the protein in its structure may result in the protein to lose its therapeutic activity. Similarly, degradation or structural changes of the immunogenic composition will also lead to a reduction in the effectivity of the composition in inducing and/or boosting an immune response in an individual. An immunogenic composition is preferably administered to an individual to counteract or prevent a viral or bacterial infection. Protein contained within the aqueous immunogenic composition can be a single protein or a multimeric protein or part of a complex comprising said protein (e.g. such as part of a virus or a cell, e.g. bacterial cell).

In a preferred embodiment said protein or protein complex comprises a live attenuated, inactivated or mutated virus or bacterium or an immunogenic viral or bacterial protein or an immunogenic part of such protein (e.g. an immunogenic peptide), split virus or bacterium, or whole bacterial cells. If said immunogenic composition is administered to provide protection against a viral or bacterial infection, degradation of protein may result in loss of protective capability of the immunogenic composition. As used herein a "live attenuated" virus or bacterium is a virus or bacterium that is less pathogenic than the wild-type virus or bacterium, but that has maintained immunogenic properties. As used herein an "inactivated" virus or bacterium refers to a virus or bacterium which has been inactivated so that it is no longer infectious, while immunogenic properties have been maintained at least in part. An inactivated virus or bacterium may be in the form of inactivated whole virus or bacterial cells. However, inactivation may result in disruption of viruses or bacterial cells. Therefore, inactivated virus or bacterium may also be in disrupted form. As used herein, a "split" virus or bacterium refers to virus or bacterium that is disrupted using for instance a detergent.

The aqueous composition of the invention may comprise a reactive compound, such as formaldehyde, which is generally present to inactivate a

virus or bacterium. Additional or alternative reactive compound may also be present to inactivate any residual formaldehyde in the aqueous solution, such as the reactive compound sulphite. As detailed above, heavy metals are thought to catalyse oxidation of (bi)sulphite and formation of sulphite radicals present in the aqueous composition, which in turn may induce degradation of protein present in the composition. Therefore, compositions comprising inactivated virus or bacteria or immunogenic parts thereof are likely to comprise one or more reactive compounds. Hence, the absence of heavy metals or presence below levels as identified by the present invention, is of particular relevance when the aqueous composition of the invention comprises an inactivated virus or bacterium or immunogenic part thereof. In a particularly preferred embodiment, an immunogenic composition according to the invention therefore comprises an inactivated virus. In a preferred embodiment, the aqueous composition, preferably immunogenic composition, according to the invention comprises inactivated virus or bacterium. Such virus or bacterium is for instance inactivated by a reactive compound such as formaldehyde as described by the present invention.

In another preferred embodiment, the aqueous composition, immunogenic composition or vaccine according to the invention comprises a toxoid. As used herein a “toxoid” refers to an inactivated bacterial toxin, such as an exotoxin as a result of which the toxicity is reduced or abolished while immunogenic properties are at least in part maintained. Such toxin is for instance inactivated by a reactive compound such as formaldehyde as described by the present invention. Examples of toxoids present in an immunogenic composition according to the invention include, but are not limited to, diphtheria, tetanus and botulism toxins.

The term "immunogenic viral or bacterial protein" refers to a viral or bacterial protein which is capable of eliciting an immune response. The term "immunogenic part" as used herein refers to a part of a viral or bacterial protein which is capable of eliciting an immune response. Preferably the

immune response elicited recognizes both said part of the protein and the entire protein. Therefore, in one embodiment, an aqueous composition comprising a protein according to the invention is an immunogenic composition. Said composition is preferably a therapeutic composition and/or prophylactic composition such as a vaccine. Also provided is a vaccine that is an aqueous composition comprising a protein according to the invention

An “immunogenic composition” is herein defined as a composition that is capable of eliciting an immune response when administered to an individual. The elicited immune response can be humoral, cellular or a combination thereof and includes, but is not limited to, the production of antibodies, B cells such as activated B cells, and T cells such as activated T cells. An immune response as used herein is preferably directed specifically to one or more immunogens within a composition comprising a protein according to the invention. An immunogenic composition of the present invention can be administered to an individual by any technique known in the art including, but not limited to, intramuscular (IM), intradermal (ID), subcutaneous (SC), intracranial (IC), intraperitoneal (IP), or intravenous (IV) injection, transdermal, oral, intranasal, or rectal administration, and combinations thereof, preferred are intramuscular (IM), intradermal (ID), subcutaneous (SC), intracranial (IC), intraperitoneal (IP), or intravenous (IV) injection. In a preferred embodiment an immunogenic composition comprising a protein according to the invention is used for eliciting an immune response that may be useful in chronic setting (such as cancer treatment) or prophylactic setting (such as a typical vaccine). It is preferred that the immunogenic composition is used as a vaccine, i.e. prophylactic use. In this embodiment the aluminium is typically present as the adjuvant.

An "adjuvant" as used herein refers to a pharmacological or immunological agent that modifies the effect of other agents, such as an immunological agent that increases the antigenic response. Adjuvants typically serve to bring the antigen—the substance that stimulates the specific

protective immune response—into contact with the immune system and influence the type of immunity produced, as well as the quality of the immune response (magnitude or duration); decrease the toxicity of certain antigens; and provide solubility to some vaccines components

An "individual" is herein defined as a human or an animal. Individuals include but not limited to chickens, ducks, geese, turkeys, swans, emus, guinea fowls and pheasants, humans, pigs, ferrets, seals, rabbits, cats, dogs and horses. In a preferred embodiment of the invention an individual is a mammal, preferably a human.

The "micro" in microgram or microliter or other unit is sometimes referred to as mc, the symbol µ or the letter u. A value of 3 mcgram is thus 3 microgram, 3 µl is 3 microliter and 3 ugram is 3 microgram.

An aluminium adjuvant is often prepared by controlled exposure of an aqueous solution of aluminium ions, to alkaline conditions (for review see, Lindblad, EB (2004) Immunol. and Cell Biol. Vol 82: 497-505). In the present invention it has been found for the JEV product (see experimental part) that a large amount of the heavy metal in this aqueous solution of aluminium ions ends up in the aluminium salt precipitate for the aluminium adjuvant. It has further been found that the amount of heavy metal in the aluminium precipitate affects the stability of the vaccine during storage of the vaccine. The amount of heavy metal that is present in the aluminium-salt can thus be controlled by determining the amount of heavy metal in the salt but also, and preferably, by controlling the amount of heavy metal in the aqueous solution of aluminium ions. The invention thus further provides a method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ion and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, preferably wherein said solution and/or the aluminium-salt

precipitate is determined to comprise an amount that results in less than 350ppb heavy metal in the final composition e.g. when re-suspended in the final composition. In a preferred embodiment, the invention provides a method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, wherein the precipitate is selected that is able to provide an aqueous composition comprising between 5 mcgram/ml and 50 mg/ml aluminium, preferably between 0,1 mg/ml and 2,5 mg/ml, and comprising no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content, wherein said composition comprises no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content, no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content, and/or no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content. In a preferred embodiment, said the precipitate comprises no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content, no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content, no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content, and no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content.

Also provided is a pharmaceutical composition comprising a protein according to the invention, optionally further comprising a pharmaceutically acceptable carrier and/or diluent. “A pharmaceutically acceptable diluent” as used herein is defined as any solution, substance or combination thereof that has no biologically or otherwise unwanted activity, meaning that it can be

administered to an individual together with other components of an immunological composition without causing a substantial adverse reaction. Examples of suitable carriers for instance comprise keyhole limpet haemocyanin (KLH), serum albumin (e.g. BSA or RSA) and ovalbumin. In one preferred embodiment said suitable carrier comprises a solution, like for example saline.

In one aspect, a method according to the invention is used for prolonging the storage life or shelf life of an aqueous composition comprising a protein according to the invention. As used herein, the term "shelf life" is defined as the period of time a composition comprising a protein according to the invention can be stored without becoming unsuitable for use, for instance due to degradation of protein (e.g. is within the potency specification of the composition, e.g. vaccine, as required by the regulatory agency that approved or will approve the vaccine). During storage of aqueous compositions as described herein, degradation of the protein may occur, in particular when a certain level (as described herein) of heavy metals is exceeded. Degradation generally increases with time when such aqueous compositions are stored. Now that it is found that degradation of protein is reduced in an aqueous composition comprising in addition to said protein an aluminium-salt, if said composition comprises less than 350 ppb heavy metal based on the weight of the aqueous composition, it has become possible to counteract degradation of protein in aqueous compositions. By counteracting degradation of protein with a method according to the invention, the stability of said protein within said composition is increased and the storage life of an aqueous compositions comprising said protein is prolonged. An aqueous composition according to the present invention provides the advantage that it is stable and does not undergo degradation of protein during a prolonged period. Such aqueous composition comprising a protein according to the invention is stable for at least one month at elevated temperature such as e.g. 20 or 37°C, preferably for at least three months at elevated temperature such as e.g. 20 or 37°C.

An aqueous composition comprising a protein according to the invention is preferably stored at a temperature of between 0 °C and 20 °C to contribute to an increased shelf life, more preferably between 2 °C and 15 °C, more preferably between 2 °C and 10 °C, most preferably between 2 °C and 8 °C. The shelf life at temperature between 2 °C and 8 °C is stable preferably for at least three months such as e.g. 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 months, preferably 20 or 24 months, more preferably 20 months at a temperature of between 2 to 8°C degrees Celsius. Provided is thus an aqueous composition comprising a protein according to the invention having a shelf life of around 12 to 24 months. The invention further provides an aqueous solution according to the invention which has been stored for at least one month, preferably for at least two months, more preferably for at least three months, more preferably for at least six months. An aqueous composition preferably comprises a protein and an aluminium salt, said composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition, between 5 mcgram/ml and 50 mg/ml aluminium, preferably between 0,1 mg/ml and 2,5 mg/ml, and no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content, wherein said composition comprises no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content, no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content, and/or no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content. In a preferred embodiment, said composition comprises no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content, no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content, no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content, and no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content.

As used herein "a stable protein composition" means that when compared to a starting composition not more than 50%, preferably not more than 40%, even more preferably not more than 30%, even more preferably not more than 20%, even more preferably not more than 10, even more preferably not more than 5% of the protein in said composition is degraded. "Degraded" in this context refers to any detectable modification of the protein when compared to the protein in the starting composition. For instance, a decrease in detection of the protein with a monoclonal antibody such as e.g. an antibody recognizing a neutralizing epitope is suitable and can be measured with any method known in the art, such as ELISA (see Example 4). The level detected can for instance be compared with the level detected with a polyclonal specific for the same protein such as e.g. representing the amount of total protein (changed or unchanged). Also provided is a method of prolonging the shelf life of an aqueous composition comprising a protein and an aluminium-salt, said method comprising selecting and/or preparing an aluminium-salt resulting in an aqueous composition with a heavy metal content of less than 350ppb based on the basis of weight of the aqueous composition and combining said aluminium salt, said protein and water to produce said aqueous composition.
Further provided is a method of improving the shelf life reproducibility of preparations of aqueous compositions comprising a protein and an aluminium-salt, said method comprising obtaining at least two different aluminium-salt preparations, determining the amount of at least one heavy metal in said aluminium-salt preparations, selecting from said aluminium-salt preparations, aluminium-salt preparations that comprise less than 350ppb of said at least one heavy metal; and combining aluminium salt of said selected preparations with said protein and water to produce said aqueous compositions. Preferably selected is a composition comprising between 5 mcgram/ml and 50 mg/ml aluminium, preferably between 0,1 mg/ml and 2,5 mg/ml, and comprising no more than 700 ppm of heavy metal and preferably no more than 450 ppm

heavy metal when compared to the aluminium content, wherein said composition comprises no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content; no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content; and/or no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content. More preferably, said composition comprises no more than 700 ppm of heavy metal and preferably no more than 450 ppm heavy metal when compared to the aluminium content, wherein said composition comprises no more than 420 ppm Fe, preferably no more than 100 ppm, when compared to the aluminium content; no more than 18 ppm, preferably no more than 3 ppm, of Ni when compared to the aluminium content; and no more than 2.5 ppm of Cu, preferably no more than 0.5 ppm, when compared to the aluminium content.

In another aspect the invention provides a method for analysing the storage stability of a composition comprising aluminium and a therapeutic or prophylactic compound, said method comprising combining into a composition a pre-determined amount of therapeutic or vaccine and a pre-determined amount of an aluminium salt, said method further comprising storing said composition for at least 2 weeks, preferably at least 4 weeks and preferably at least one month, at a temperature of more than 20°C, preferably at a temperature of about 22 °C and determining the stability and/or the amount of protein, preferably therapeutic or prophylactic compound, in said composition. As demonstrated in Examples 1 and 2, a temperature of 22 °C, which is higher temperature compared to normal storage conditions or about 2-8°C, results in accelerated degradation of protein in an aqueous composition comprising protein, an aluminium-salt and more than 350ppb of said heavy metal based on weight with respect to said composition. Thus, a temperature of about 22 °C and a storage duration of at least 2 weeks, preferably at least 4 weeks, are suitable to determine the storage stability of aqueous compositions comprising a protein according to the invention. The storage stability can be determined

by any method known in the art. The storage stability of an aqueous composition comprising a protein according to the invention is preferably analyzed by determining the storage stability of said protein, preferably by determining a storage sensitive epitope on said protein. For instance, as described in Example 1 and 2, the stability of a protein, preferably an antigen, is determined by determining the ratio of intact storage sensitive epitope, such as intact antigenic epitope (e.g. epitope of a neutralizing epitope) content, and total protein content. "Intact storage sensitive epitope" or "intact antigenic epitope" as used herein means that degradation has not occurred within said epitope. The intact antigenic epitope content is for instance measured by determining protein bound to a monoclonal antibody specifically directed against said epitope in, for example, an ELISA. The total protein content is for instance measured by determining protein bound to polyclonal antibody which is directed against various epitopes within the protein, for example by ELISA. The relative specific epitope content can then be expressed as the ratio of the total antigen content determined by binding to monoclonal antibody divided by total antigen content determined by binding to polyclonal antibody. A high ratio indicates high antigenic epitope content and a low ratio indicates a low antigenic epitope content. A low ratio measured for an aqueous composition after storage at least 20°C, preferably 22°C, for at least 2 weeks, preferably 4 weeks, as compared to the ratio measured for said aqueous composition before storage, indicates that structural changes have taken place within the antigenic epitope. Structural changes within said antigenic epitope indicate reduced storage stability of the aqueous composition.

The heavy metal content of an aqueous composition prepared according to the invention is less than 350 ppb based on the weight of the aqueous composition. Generally, the less heavy metal such aqueous composition contains, the less degradation of protein occurs. Preferably, therefore, the heavy metal content is less than 325 ppb based on the weight of the aqueous composition, more preferably less than 300 ppb, more preferably less than 275,

more preferably less than 250 ppb and more preferably less than 235 ppb based on the weight of the aqueous composition.

As used herein the term "heavy metal" refers to the total amount of elements that exhibit metallic properties and includes the transition metals, metalloids, lanthanides, and actinides. Transition metals are elements whose atom has an incomplete d sub-shell, or which can give rise to cations with an incomplete d sub-shell, and include zinc, molybdenum, cadmium, scandium, titanium, technetium, palladium, vanadium, chromium, manganese, iron, cobalt, rhodium, hafnium, copper, nickel, ytrrium, niobium, ziorconium, rughenium, silver, tantalum, rhenium, thungsten, osmium, meitnerium, platinum, iridium, mercury, bohrium, seaborgium, hassium. Metalloids are Boron (B), Silicon (Si), Germanium (Ge), Arsenic (As), Antimony (Sb), Tellurium (Te), Polonium (Po). The lanthanides are the fifteen metallic chemical elements with atomic numbers 57 through 71, i.e. Lanthanum, Cerium, Praseodymium, Neodymium, Promethium, Samarium, Europium, Gadolinium, Terbium, Dysprosium, Holmium, Erbium, Thulium, Ytterbium and Lutetium. The actinides are the fifteen metallic chemical elements with atomic numbers from 89 to 103, actinium, thorium, protactinium, uranium, neptunium, plutonium, americium, curium, berkelium, californium, einsteinium, fermium, mendelevium, nobelium and lawrencium. The heavy metal is preferably a metal of the D-block of the periodic table. The heavy metal is preferably a metal of group 3-12 of the periodic table including the lanthanides and the actinides. In a particularly preferred embodiment the heavy metal is a metal of the D-block, or group 3-12 of the periodic table and not including the lanthanides and the actinides. Preferably said heavy metal is an element selected from the transitional metals. In another preferred embodiment the heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr, Pb, Rb and Mo. In another preferred embodiment, said heavy metal is a metal having a molar mass of between 21 and 83, preferably said heavy metal is a metal of the D-block of the periodic table with a molecular mass of

between 21 and 83, more preferably from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr and Mo. Preferably said heavy metal is a metal from the D-block of the periodic table with a molecular mass of between 21-30 and 39-48, more preferably from Cu, Ni, Co, Ru, Cd, Ag, Fe, V, Cr and Mo In an even more preferred aspect, the heavy metal is selected from the heavy metals Cu, Ni, and Fe.

As demonstrated in the Examples, Aluminium hydroxide (Alum) Lot 4230 was identified to contribute significantly to antigen degradation in JEV vaccine FVL09L37. In Example 3 it is shown that this Alum lot comprises at least the following metals: Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V. Higher levels of Fe, Ni and Cu ions were noted in Alum lot 4230 when compared to other investigated lots. Lot 4230 was the only one where residual Cu ions were detected. Therefore, preferably said heavy metal is selected from the group consisting of Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, more preferably from Fe, Ni and Cu.

The amount of heavy metal in the aqueous composition is preferably less than 350 ppb based on the weight of the aqueous composition. Preferably the amount of heavy metal in the aqueous composition is less than 250 ppb, preferably less than 225, more preferably less than 200, more preferably less than 150, more preferably less than 100, more preferably less than 50, more preferably less than 25 ppb based on the weight of the aqueous composition. The amount of heavy metal in an aqueous composition of the invention is defined herein above. This amount is typically for the total of determined heavy metals, or for the heavy metals Fe, Cr and Ni, or a combination thereof which constitute the major heavy metals by weight in the aqueous composition of the invention. For specific heavy metals different maximums amounts may be preferred. For instance, it is preferred that the amount of Fe in the aqueous composition of the invention is less than 350 ppb based on the weight of the aqueous composition. In a preferred embodiment the amount of Fe is less than

250 ppb, preferably less than 210 ppb Fe based on the weight of the aqueous composition.

There is strong evidence that many of the pro-inflammatory effects of aluminium adjuvants are mediated via the formation of reactive oxygen species (ROS). Aluminium can, under physiological conditions, promote the reduction of Fe(III) to Fe(II) and the oxidation of the latter. Thus the combination of Fe and Al in the adjuvant will potentiate the formation and activities of ROS (Exley, C (2010). Trends in Immunol. Vol. 31: pp 103-109). In the present invention it has been found that Fe can be present in an aqueous composition of the invention without significantly affecting the storage stability of the composition. In this embodiment of the invention it is preferred that the aqueous composition of the invention comprises between 5 ppb and 250 ppb Fe based on the weight of the aqueous composition. In these amounts the formation of ROS during storage due to the presence of such amount of Fe (if any ROS) does not significantly affect the storage stability of the aqueous composition as defined elsewhere herein. However, the amounts are sufficient to allow pro-inflammatory effects following administration of the vaccine in vivo.

In the present invention it has been found that particularly the presence of heavy metal Cu seriously affects the storage stability of the composition of the invention. The amount of Cu in a protein containing composition or method for producing the protein composition of the invention is preferably less than less than 25 ppb. Preferably less than 5 ppb, more preferably less than 1 ppb, more preferably less than 0,2 ppb based on the weight of the aqueous composition. In a particularly preferred embodiment an aqueous composition of the invention therefore comprises less than 3 ppb Cu based on the weight of the aqueous composition. Preferably less than 2,5 ppb. The amount of Cu in a composition or method of the invention is in a particularly preferred embodiment less than 1,25 ppb based on the weight of the aqueous composition. In a particularly preferred embodiment said aqueous composition

comprises Cu at a level that is below the detection limit of the method for the detection of copper as described in the Examples.

In the present invention it has been found that particularly the heavy metal Ni affects the storage stability of the composition of the invention. The amount of Ni in a composition or method of the invention is preferably less than 200 ppb. Preferably less than 40 ppb, preferably less than 9 ppb and preferably less than 2 ppb based on the weight of the aqueous composition. In a particularly preferred embodiment an aqueous composition of the invention therefore comprises less than 40 ppb Ni based on the weight of the aqueous composition. Preferably less than 30 ppb, more preferably less than 20 pbb and more preferably less than 15 pbb Ni based on the weight of the aqueous composition. In a particularly preferred embodiment said aqueous composition comprises Ni at a level that is below the detection limit of the method for the detection of nickel as described in the Examples.

The heavy metal can be present in electronic neutral form or it can be ionised. Typically and preferably the heavy metal is present in ionic form in an aqueous composition of the invention.

Metal content of a composition can be determined in various ways. In one aspect, a method according to the invention comprises determining the level of a heavy metal in an aqueous composition and/or the aluminium-salt present in said aqueous composition. Methods for measuring the level of one or more heavy metals in an aqueous solution are known in the art. Examples of such methods include mass spectrometry, such as inductively-coupled-plasma mass spectrometry (ICP-MS), flame atomic absorption spectrometry (F-AAS), and/or graphite furnace atomic absorption spectrometry (GF-AAS).

An example of an assay which can be used to determine the content of heavy metals is described in Example 3. The assay involves treating a sample of an aqueous solution containing an Aluminum-salt with concentrated HNO_3 under heat until a clear solution is obtained. The clear solution can then be further diluted and analyzed, for instance by ICP-MS, F-AAS and/or GF-AAS.,

for the presence and content of metal ions including Pb, Cd, Cr, Co, Fe, Cu, Ni, Ag, W and Al.

Examples 1 and 2 demonstrate that the JEV antigen shows higher stability at pH 7.5-8 as compared to pH 7. In the Examples antigen stability is expressed as the ratio of monoclonal/polyclonal ELISA. The monoclonal antibody used (clone 52-2-5) was shown to recognize a neutralizing epitope in the Japanese Encephalitis Vaccine (JEV). The relative specific epitope content can be expressed as the ratio of the total antigen content determined by "monoclonal ELISA" divided by total antigen content determined by "polyclonal ELISA". Without being bound to theory, the effect of a higher antigen stability at pH 7.5-8 can be explained based on the underlying assumed complex reaction chemistry of sulphites. pH might influence the related to equilibrium reaction conditions of the sulphite / formaldehyde reaction and surface charge of certain proteins/amino acid side chains accessible to modification. The pH may affect oxidation by direct influence on redox potentials of the amino acid residues and the oxidizing agents, e.g. free radicals. Therefore, in one embodiment, a method according to the invention comprises buffering said aqueous composition at a pH of between 7.5 and 8.5.

Various aluminium salts are being used in compositions for administration of an individual. Aluminium adjuvant typically contains an aluminium oxide or sulphate or a combination thereof. In a preferred embodiment the aluminium salt comprises aluminiumoxide (Al_2O_3), aluminiumhydroxide ($Al(OH)_3$) or aluminiumphosphate ($AlPO_4$).

In a preferred embodiment the aqueous composition of the invention further comprises a reactive compound. Typically though not necessarily the reactive compound is present as a result of a manipulation of the aqueous composition, for instance to treat or inactivate infectious agent if any in the composition. The reactive compound can also be present for another reason. Sulphite, for instance, is sometimes present to inactivate any residual

formaldehyde in the aqueous solution. Formaldehyde is typically a chemical that is often used to inactivate any infectious agent.

The reactive compound is preferably a redox active compound, radical building compound and/or a stabilizing compound. In a preferred embodiment said aqueous composition of the invention comprises formaldehyde, ethanol, chlorophorm, trichloroethylene, acetone, triton-X-100, deoxycholate, diethylpyrocarbonate, sulphite, $Na_2S_2O_5$, beta-proprio-lacton, polysorbate such as Tween 20®, Tween 80®, O_2, phenol, pluronic type copolymers, or a combination thereof.

Sulphite is preferably present in an amount of between 0,1 mM and 5 mM, or preferably between 0,5-2mM. Formalin is preferably present in an amount of between 0,1 mM and 5 mM, more preferably between 0,5 mM and 2mM. Oxygen is preferably present in an amount that is equivalent to the solubility of O_2 at the measured temperature, O_2 is preferably present in an amount of between 10 and 250 uM when measured at 20 degrees Celsius. When measured at a temperature of 0 degrees Celsius O_2 is preferably present in an amount of between 10 and 400 uM. A stabilizing compound is preferably present in an amount of between10 and 400 uM. Similarly a redox active compound is present in an amount of between 0,1 mM and 5 mM, or preferably between 0,5-2mM. A radical building compound is preferably present in an amount of between 0,1 mM and 5 mM, or preferably between 0,5-2mM. In this context and for the sake of clarity it is important to note that redox active compound, the radical building compound and/or the stabilizing compound is consumed in the production of a radical, whereas the heavy metal is indicated herein above, is a catalyst in the production of a radical and is not consumed, as such. The redox active compound, the radical building compound and/or the stabilizing compound is therefore not a heavy metal.

The total amount of redox active compound, the radical building compound and/or the stabilizing compound although small in absolute amounts can still be significant in relation to the antigen or protein in the

aqueous composition of the invention. The antigen/protein is preferably present in an amount of between 0,1 nmol to 1 umol, more preferably between 1 nmol and 100 nmol.

The concentration of protein, preferably a therapeutic or vaccine protein, in an aqueous composition comprising a protein according to the invention is preferably between 1 ng/ml and 10 mg/ml, preferably between 10 ng/ml and 1 mg/ml, more preferably between 100 ng/ml and 100 mcg/ml, such as between 1 mcg/ml and 100 mcg/ml. The concentration is preferably at least 1 ng/ml to ensure that the therapeutic or vaccine protein is in a concentration sufficient to exert its therapeutic effect when administered to an individual. The concentration should, however, preferably not exceed 10 mg/ml in order to prevent or reduce the occurrence of possible side effects associated with administration of said protein to an individual. In particular, the concentration of viral protein in an aqueous composition according to the invention comprising JEV is preferably between 0.01 μg/ml and 1 mg/ml, more preferably between 0.1 mcg/ml and 100 mcg/ml. In an exemplary embodiment, of the invention, an aqueous composition according to the invention comprises about 10 mcg/ml of JEV. The dose of a single administration of an aqueous composition comprising a protein, preferably a therapeutic or vaccine protein, according to the invention is preferably between 0.1 ml and 10 ml, preferably between 0.5 ml and 5 ml, such as 0.5 ml, 1 ml, 1.5 ml, 2 ml, 2.5 ml, because such dose allows for convenient administration to an individual, such as a human.

The aqueous composition according to the invention may further comprise a nucleic acid molecule. The nucleic acid may be administered for therapeutic purposes. In that case, the aqueous composition according to the invention preferably comprises a nucleic acid molecule, such as a plasmid comprising the nucleic acid sequence encoding an antigen or antigens or virus or bacterium or immunogenic part thereof against which an immune response is sought. Incorporation of such nucleic acid molecule relies on the in situ production of

the target antigen, virus, bacterium or immunogenic part thereof. In another preferred embodiment, the aqueous composition according to the invention comprises a nucleic acid in the form of an antisense RNA, RNAi or mimic thereof. In yet another embodiment, the aqueous composition according to the invention comprises the nucleic acid in the form of an infectious agent as such, a virus or a modified virus, as is the case in many gene therapy approaches. The nucleic acid concentration in the aqueous composition, immunogenic composition or vaccine according to the invention is preferably in the range of about 1 ng/ml to about 10 mg/ml, preferably about 0.1 mcg/ml to about 1 mg/ml, more preferably about 1 mcg/ml to about 100 mcg/ml. The suitable dosage will depend upon the subject to which the composition is administered and the size of the nucleic acid sequences present in the composition.

An aqueous composition according to the invention may further comprise a polysaccharide and/or an oligosaccharide, preferably the polysaccharide and/or oligosaccharide capsule of encapsulated bacteria against which an immune response is sought. Examples of polysaccharides and oligosaccharides that can be present in an aqueous composition according to the invention, include but are not limited to pneumococcal polysaccharides, meningococcal polysaccharides, *Haemophilus influenzae* type b polysaccharide, streptococcal group B polysaccharides, *Salmonella typhi* Vi polysaccharide, polysaccharides or oligosaccharides derived from streptococcus group A, *Staphylococci, Neisseria meningitidis*, *Klebsiella pneumoniae*, *Enterococci, E. coli, Pseudomonas aeruginosa*, and *Bacillus anthracis*. Such polysaccharide and/or an oligosaccharide may be present in the aqueous composition according to the invention as such, or, alternatively, the polysaccharide and/or an oligosaccharide may be conjugated to a protein. The polysaccharide and/or oligosaccharide concentration in the aqueous composition, immunogenic composition or vaccine according to the invention is preferably in the range of about 10 ng/ml to about 500 mcg/ml, more preferably about 0.1 mcg/ml to about 500 mcg/ml, more preferably about 1 mcg/ml to about 50 mcg/ml. The

suitable dosage will depend upon the subject to which the composition is administered.

A method according to the invention is preferably used to increase stability of an immunogenic composition, preferably a vaccine, comprising an aluminium-salt based adjuvant. Non-limiting examples of such vaccines are those directed against infection with *Bacillus anthracis* (causing Anthrax), *Corynebacterium diphtheriae* (causing diphteria), *Clostridium tetani* (causing tetanus), Pseudomonas such as *Pseudomonas aeruginosa*, Staphylococcus such as *Staphylococcus aureus* or *Staphylococcus epidermidis*, Haemophilus influenzae type B bacteria (Hib), polio virus, hepatitis A virus, hepatitis B virus, Human Papillomavirus, influenza virus, Japanese encephalitis virus, Rotavirus, *Rickettsiae* bacteria (causing Typhus), yellow fever virus, Varicella Zoster Virus, Meningococcus, or combinations thereof, such as, but not limited to, DTP (diphteria, tetanus, polio). An aqueous composition comprising a protein according to the invention therefore preferably comprises a protein which is a viral or bacterial protein, preferably a protein of *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Clostridium tetani*, Haemophilus influenzae type B bacteria (Hib), polio virus, hepatitis A virus, hepatitis B virus, Human Papillomavirus, influenza virus, Japanese encephalitis virus, Rotavirus, *Rickettsiae* bacteria, yellow fever virus, Varicella Zoster Virus and/or Meningococcus. In a preferred embodiment, said protein contained in a composition comprising a protein according to the invention is a viral protein from a virus of the Flaviviridae family, preferably of a Japanese encephalitis virus (JEV). As demonstrated in the Examples, the stability of aqueous compositions comprising a JEV protein, an aluminium-salt and comprising less than 350 ppb heavy metal based on the weight of the aqueous composition, and in particular wherein the amount of Cu is less than 3 ppb based on the weight of the aqueous composition, is increased as compared to aqueous composition comprising more than 350 ppb of heavy metal and more than 3 ppb of Cu. Thus, a method according to the invention is particularly suitable to

increase stability of an aqueous composition comprising a JEV protein. In tables 30 and 31, a non-limiting list of examples of aluminium based vaccines, both for human use and for veterinary use, is given. A method according to the invention is particularly suitable to increase stability of a vaccine listed in table 30 and/or table 31. Therefore, a vaccine listed in table 30 and/or table 31 is preferably prepared using a method of the invention.

In another preferred embodiment or aspect of the invention, said protein contained in a composition comprising a protein according to the invention is a bacterial protein from a bacterium of the Pseudomonas family, preferably of *Pseudomonas aeruginosa.* As demonstrated in the Examples, the stability of aqueous compositions comprising *Pseudomonas aeruginosa* fusion protein (SEQ ID NO: 1) and an aluminium-salt is reduced when more than 350 ppb heavy metal based on the weight of the aqueous composition is present.

An aqueous composition comprising a protein according to the invention is particularly suitable for use as an immunogenic composition or vaccine. For instance, such compositions are particularly useful for immunize an individual to treat or prevent a viral or bacterial infection. In one embodiment, the invention therefore provides a method for the treatment of an individual comprising obtaining an immunogenic aqueous composition comprising a protein and an aluminium-salt, said aluminium-salt having less than 350 ppb heavy metal based on the weight of the aqueous composition, preferably less than 3 ppb of Cu, and administering the immunogenic aqueous composition to an individual in need thereof. Also provided is a method for the prophylactic treatment of an individual comprising obtaining an immunogenic aqueous composition comprising a protein and an aluminium-salt, said aluminium-salt having less than 350 ppb heavy metal based on the weight of the aqueous composition, preferably less than 3 ppb of Cu, and administering the immunogenic aqueous composition to an individual in need thereof. Further provided is a method for inducing and/or boosting an immune response towards an antigen in an individual, said method comprising obtaining an

aqueous composition comprising a protein comprising said antigen and an aluminium-salt, said aluminium-salt having less than 350 ppb heavy metal based on the weight of the aqueous composition, preferably less than 3 ppb of Cu, and administering the aqueous composition to an individual in need thereof. In another aspect the invention provides a method for immunizing an individual comprising administering to said individual at least two immunogenic compositions at an interval of at least two weeks between each administration, and wherein each of said at least two immunogenic compositions comprise the same antigen, and wherein at least one of said immunogenic compositions further comprises an aluminium salt having less 350 ppb heavy metal based on the weight of the aqueous composition, preferably less than 3 ppb of Cu, and administering the immunogenic aqueous composition to an individual in need thereof. Nucleic acid compositions are sometimes also administered together with aluminium. Thus for the present invention it is possible to replace "protein" in an aqueous composition of the invention with nucleic acid. Thus in one embodiment the invention provides a method for preparing an aqueous composition comprising aluminium and a nucleic said method comprising

- combining an aluminium-salt, said nucleic acid and water to produce said aqueous composition and
- determining the level of a heavy metal in the aqueous composition and/or the aluminium-salt.

The invention also provides a method for preparing an aqueous composition comprising aluminium and a nucleic acid said method comprising

- preparing or selecting an aluminium-salt having less 350 ppb heavy metal based on the weight of the final aqueous composition, preferably less than 3 ppb of Cu, and administering the immunogenic aqueous composition to an individual in need thereof and
- combining said aluminium salt, said nucleic acid and water to produce said aqueous composition.

In a preferred embodiment said methods further comprising buffering said aqueous composition at a pH of between 7.5 and 8.5. In a particularly preferred embodiment said methods, further comprise packaging aliquots of said aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition in separate air-tight storage containers. The nucleic acid may be administered for therapeutic purposes. For instance, in the form of an antisense RNA, RNAi or mimic thereof. The nucleic acid may also be administered in the form of an infectious agent, typically a virus or a modified virus, as is the case in many gene therapy approaches. In that case the nucleic acid is enclosed in a particle that comprises protein. The invention thus further provides an aqueous composition comprising a nucleic acid and an aluminium-salt, said composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition. The nucleic acid concentration in the aqueous composition, immunogenic composition or vaccine according to the invention is preferably in the range of about 1 ng/ml to about 10 mg/ml, preferably about 0.1 mcg/ml to about 1 mg/ml, more preferably about 1 mcg/ml to about 100 mcg/ml. The appropriate dosage will depend upon the subject to which the composition is administered and the size of the nucleic acid sequences present in the composition.

Compositions comprising polysaccharide, oligosaccharide or polysaccharide-polypeptide conjugate are sometimes also administered together with aluminium. Thus for the present invention it is possible to replace “protein” in an aqueous composition of the invention with polysaccharide, oligosaccharide or polysaccharide-polypeptide conjugate or a combination thereof. Such polysaccharide or, oligosaccharide is preferably the polysaccharide or oligosaccharide of the capsule of encapsulated bacteria against which an immune response is sought. Examples of polysaccharides and oligosaccharides that can be present in an aqueous composition according to the invention, include but are not limited to pneumococcal polysaccharides, meningococcal polysaccharides, *Haemophilus influenzae* type b polysaccharide,

streptococcal group B polysaccharides, *Salmonella typhi* Vi polysaccharide, polysaccharides or oligosaccharides derived from streptococcus group A, *Staphylococci, Neisseria meningitidis*, *Klebsiella pneumoniae*, *Enterococci, E. coli, Pseudomonas aeruginosa*, and *Bacillus anthracis*. Such polysaccharide and/or an oligosaccharide may be present in the aqueous composition according to the invention as such, or, alternatively, the polysaccharide and/or an oligosaccharide may be conjugated to a protein. Thus in one embodiment the invention provides a method for preparing an aqueous composition comprising aluminium and a polysaccharide or oligosaccharide method comprising

- combining an aluminium-salt, said polysaccharide or oligosaccharide and water to produce said aqueous composition and

- determining the level of a heavy metal in the aqueous composition and/or the aluminium-salt.

The invention also provides a method for preparing an aqueous composition comprising aluminium and a polysaccharide or oligosaccharide said method comprising

- preparing or selecting an aluminium-salt having less 350 ppb heavy metal based on the weight of the final aqueous composition, preferably less than 3 ppb of Cu, and administering the immunogenic aqueous composition to an individual in need thereof and

- combining said aluminium salt, said polysaccharide or oligosaccharide and water to produce said aqueous composition.

The polysaccharide and/or oligosaccharide concentration in the aqueous composition, immunogenic composition or vaccine according to the invention is preferably in the range of about 10 ng/ml to about 500 µg/ml, more preferably about 0.1 mcg/ml to about 500 mcg/ml, more preferably about 1 mcg/ml to about 50 mcg/ml. The appropriate dosage will depend upon the subject to which the composition is administered.

The polysaccharide-polypeptide conjugate according to the present invention comprises at least one polysaccharide and at least one polypeptide. The polysaccharide according to the invention is preferably a bacterial capsular polysaccharide. Capsular polysaccharides can be prepared by standard techniques known to those of skill in the art.

In an embodiment, the polysaccharide is a S. pneumoniae capsular polysaccharide. In another embodiment, the S. pneumoniae capsular polysaccharide is selected from the group consisting of serotype 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 1OA, HA, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, representing the 23 pneumococcal serotypes causing the vast majority of pneumococcal disease in all age groups, out of the more than 90 serotypes known so far.

The term "polysaccharide" as used herein refers to polysaccharides and/or oligosaccharides. Polysaccharides are isolated from bacteria and may be depolymerized to a preferred size range by known methods (see for example EP 497524 and EP 497525). Oligosaccharides have a low number of repeat units (typically 5-30 repeat units) and are typically hydrolysed polysaccharides.

Capsular polysaccharides of Streptococcus pneumoniae comprise repeating oligosaccharide units which may contain up to 8 sugar residues. For a review of the oligosaccharide units for the key Streptococcus pneumoniae serotypes see Jones et al, An. Acad. Bras. Cienc, 2005, 77(2):293-324. In one embodiment, a capsular saccharide antigen may be a full length polysaccharide, however in others it may be one oligosaccharide unit, or a shorter than native length saccharide chain of repeating oligosaccharide units. Full length polysaccharides may be "sized" or "depolymerized", i e. their size may be reduced by various methods known in the art (as described above). The term "depolymerization" includes partial depolymerization.

The depolymerization of the polysaccharides may then be followed by an activation step prior to conjugation to a carrier polypeptide. By "activation" is

meant chemical treatment of the polysaccharide to provide chemical groups capable of reacting with the carrier polypeptide. Appropriate methods are known in the art.

By "polypeptide" or "protein" is meant any chain of amino acids having at least 10 and preferably at least 100 amino acids in peptide linkage with each other, regardless of post-translational modification. Suitable polypeptide carriers include, but are not limited to, diphtheria toxin, diphtheria toxoid, CRM 197, tetanus toxoid, pertussis toxoid, E. coli LT, E. coli ST, exotoxin A, outer membrane complex c (OMPC), porin, transferrin binding protein, pneumolysis, pneumococcal surface protein A (PspA), pneumococcal adhesin protein (PsaA), ovalbumin, keyhole limpit hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD), and the like. Carrier polypeptides are preferably polypeptides that are non-toxic and non-reactogenic and obtainable in sufficient amount and purity. In a particularly preferred embodiment, the carrier polypeptide comprises a tetanus toxoid. In another preferred embodiment, the carrier polypeptide comprises a derivative of any of the above mentioned carrier polypeptides, for example a subunit or a mutated version of E. coli LT, such as LT or the A subunit of LT (LTA) having an amino acid substitution at the position of aa 192 (e.g. LTG 192, LTT 192, LTS 192, LTA 192), LTK 63 LTR 72, or other mutants as described e.g. in WO 98/42375, WO 02/64162, US 4,761,372, US 5,308,835. The polypeptides may also contain elongations either at the carboxy- or at the amino- terminus of the polypeptide facilitating interaction with the polycationic compound(s) or the immunostimulatory compound(s).

Furthermore, the polypeptides may also be derivatized to include molecules enhancing antigen presentation and targeting of antigens to antigen presenting cells.

The polypeptide may be activated prior to conjugation.

The nature and size of the saccharide, the nature and size of protein or polypeptide, the ratio of the saccharide and protein/polypeptide, as well as

other factors and conditions for the preparation of a conjugate according to the present invention can be determined by a skilled person, as described e.g. in Robbins et al, JAMA, 1996, 276(14): 1181-5. For example, for a pneumococcus polysaccharide and a tetanus toxoid a preferred ratio is approximately 2:1.

By "conjugate" is meant a compound in which the polysaccharide is covalently linked to a carrier polypeptide. There are many conjugation reactions known in the prior art that have been employed for covalently linking polysaccharides to polypeptides in order to produce a polysaccharide-polypeptide conjugate. Three of the more commonly employed methods include: 1) reductive amination, wherein the aldehyde or ketone group on one component of the reaction reacts with the amino or hydrazide group on the other component, and the C-N double bond formed is subsequently reduced to C-N single bond by a reducing agent; 2) cyanylation conjugation, wherein the polysaccharide is activated either by cyanogens bromide (CNBr) or by l-cyano-4-dimethylammoniumpyridinium tetrafluoroborate (CDAP) to introduce a cyanate group to the hydroxyl group, which forms a covalent bond to the amino or hydrazide group upon addition of the protein component; and 3) a carbodiimide reaction, wherein carbodiimide activates the carboxyl group on one component of the conjugation reaction, and the activated carbonyl group reacts with the amino or hydrazide group on the other component. These reactions are also frequently employed to activate the components of the conjugate prior to the conjugation reaction. The polysaccharide may be conjugated to the polypeptide directly or via a linker. Linkage via a linker group may be made using any known procedure, for example, the procedures described in US 4,882,317 and US 4,695,624. Suitable linkers include carbonyl, adipic acid, B-propionamido (WO 00/10599), nitrophenyl-ethylamine (Gever et al., Med. Microbiol. Immunol, 1979, 165:171-288), haloacyl halides (US 4,057,685), glycosidic linkages (US 4,673,574; US 4,761,283; US 4,808,700), 6-aminocaproic acid (US 4,459,286), ADH (US 4,965,338), C4 to Ci2 moieties (US 4,663,160), etc.

After conjugation of the polysaccharide to the carrier polypeptide, the polysaccharide- polypeptide conjugate may be purified (enriched with respect to the amount of polysaccharide-polypeptide conjugate) by a variety of techniques known in the art. One goal of the purification step is to remove the unbound polysaccharide and/or polypeptide from the polysaccharide-polypeptide conjugate. Methods for purification include e.g. ultrafiltration in the presence of ammonium sulfate, size exclusion chromatography, density gradient centrifugation, and hydrophobic interaction chromatography.

Aluminium adjuvant, such as alumiunium hydroxide and aluminium phosphate adjuvants, are generally prepared by exposing aqueous solutions of aluminium ions, to usually slightly alkaline conditions in a well-defined and controlled chemical environment. Various soluble aluminium salts can be used for the production of aluminium hydroxide. Anions present at the time of precipitation may coprecipitate with the aluminium hydroxide or sulphate. After the precipitation of the ammonium salt by pH shift using for instance NaOH, it is not possible to remove any heavy metal present in the aluminium adjuvant. Even extensive washing does not result in a sufficient reduction of the heavy metal content. Thus, it is important to control the heavy metal before precipitation of the aluminium salt, i.e. by selection of appropriate raw materials, control of process conditions, e.g. as ammonia alum and aluminium sulfate, or other aluminium sources are in solution there should be no metal added, for instance other salts such as $CuSO_3$. Alternatively or additionally, any heavy metals present in solution is removed before precipitation of the aluminium adjuvant. This can for instance be achieved via crystallization or ion (cation) exchange, which are methods well known in the art. Ion exchange refers to a process through which (metal) ions in solution are transferred to a solid matrix which, in turn releases ions of a different type but of the same polarity into the solution. Thus the ions in solution are replaced by different ions originally present in the solid matrix. Crystallization of metals refers to

precipitation of insoluble metal crystals from metal ions in solution. Therefore in a preferred embodiment, the invention provides a method for preparing an aqueous composition comprising aluminium, a reactive compound and a protein said method comprising

- preparing an aluminium-salt that is able to provide an aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition and

- combining said aluminium salt, said reactive compound, said protein and water to produce said aqueous composition,

whereby said aluminium salt is prepared by preparing an aqueous solution of aluminium ions, removing heavy metals from said aqueous solution such as by crystallization or ion exchange, preferably cation exchange, and precipitating said aluminium-ions from said solution, preferably using a base.

Further provided is a method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions, removing heavy metals from said aqueous solution such as by crystallization or ion exchange, preferably cation exchange, and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, wherein the precipitate is selected that is able to provide an aqueous composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition.

The preparation of aluminium hydroxide from raw materials is among others described in CN101734698 and WO98/14401 which are incorporated by reference herein.

The invention further provides a method for preparing an aqueous pharmaceutical or vaccine composition comprising aluminium, a reactive compound and a protein said method comprising

- selecting an aluminium-salt that is able to provide an aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition and

- combining said aluminium salt, said reactive compound, said protein and water to produce said aqueous composition (i) having less than 350 ppb heavy metal based on the weight of the aqueous composition and (ii) comprising between 5 µg/ml and 50 mg/ml aluminium;

wherein the reactive compound is selected from the group consisting of a redox active compound, a radical building compound, a stabilizing compound and a combination of any thereof.

The method preferably further comprises buffering said aqueous composition at a pH of between 6.5 and 8.5.

The method preferably further comprises packaging aliquots of said aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition in separate air-tight storage containers.

The invention further provides a method for selecting a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, wherein the precipitate is selected that is able to provide an aqueous composition comprising (i) less than 350 ppb heavy metal based on the weight of the aqueous composition and (ii) between 5 µg/ml and 50 mg/ml aluminium.

Also provided is an aqueous pharmaceutical or vaccine composition comprising a protein, a reactive compound and an aluminium-salt, said composition comprising (i) less than 350 ppb heavy metal based on the weight of the aqueous composition and (ii) between 5 µg/ml and 50 mg/ml aluminium, wherein the reactive compound is selected from the group consisting of a redox active compound, a radical building compound, a stabilizing compound and a combination of any thereof. In a preferred embodiment said heavy metal is

selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr, Pb, Rb and Mo. The heavy metal is preferably selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V. In a particularly preferred embodiment the heavy metal is selected from Cu or Ni. The heavy metal is preferably present in ionic form. The aluminium-salt is preferably aluminiumhydroxide (Al(OH)3) or aluminiumphosphate (AlPO4).The aluminium-salt is preferably aluminiumhydroxide (Al(OH)3). The reactive compound is preferably selected from the group consisting of formaldehyde, ethanol, chlorophorm, trichloroethylene, acetone, 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol, deoxycholate, diethylpyrocarbonate, sulphite, $Na_2S_2O_5$, beta-proprio-lacton, polysorbate such as polyethylene glycol sorbitan monolaurate, polyethylene glycol sorbitan monooleate, O_2, phenol, pluronic type copolymers, and a combination of any thereof. The aqueous pharmaceutical of vaccine composition preferably comprises between 50 µg/ml and 5 mg/ml aluminium. The aqueous pharmaceutical or vaccine composition preferably comprises between 5 ppb and 250 ppb Fe based on the weight of the aqueous composition. The aqueous pharmaceutical or vaccine composition preferably comprises less than 3 ppb Cu based on the weight of the aqueous composition. The aqueous pharmaceutical or vaccine composition preferably comprises less than 40 ppb Ni based on the weight of the aqueous composition. The protein in said aqueous pharmaceutical or vaccine composition preferably is a therapeutic and/or a vaccine. The protein is preferably a viral or bacterial protein, The viral protein preferably is a protein of the Japanese encephalitis virus or a protein of the *Pseudomonas aeruginosa* bacterium. The protein is preferably a protein within a formaldehyde inactivated virus particle. The aqueous pharmaceutical or vaccine composition preferably further comprises sulphite. The invention further provides a vaccine comprising an aqueous vaccine composition according to the invention.

Where herein a range is indicated as between value X and Y, the range includes the values X and Y.

The invention further provides a method for preparing an aqueous composition comprising aluminium, a reactive compound and a protein said method comprising

- preparing or selecting an aluminium-salt that is able to provide an aqueous composition having less than 450 ppm heavy metal based on the weight of the aluminium (gram/gram) and

- combining said aluminium salt, said reactive compound, said protein and water to produce said aqueous composition. Preferably the Fe content of the aqueous composition is less than 700 ppm based on the weight of the aluminium in the composition; the Ni content is less than 18 ppm based on the weight of the aluminium in the composition; or the Cu content is less than 2,5 ppm based on the weight of the aluminium in the aqueous composition, or a combination thereof. Preferably the method further comprises buffering said aqueous composition at a pH of between 6.5 and 8.5. Preferably the method further comprises packaging aliquots of said aqueous composition having less 450 ppm heavy metal based on the weight of the aluminium in the composition in separate air-tight storage containers.

The invention further provides a method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, wherein the precipitate is selected that is able to provide an aqueous composition comprising less than 450 ppm heavy metal based on the weight of the aluminium ions (gram/gram) in the solution.

Further provided is an aqueous composition comprising a protein and an aluminium-salt, said composition comprising less than 450 ppm heavy metal based on the weight of the aluminium in the composition. The aqueous composition preferably has been stored at temperatures higher than 20°C for

at least 1 month. The heavy metal is preferably selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr and Mo. Preferably the heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V. In a particularly preferred embodiment the heavy metal is selected from Cu or Ni. Preferably the heavy metal is Cu.

The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention.

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Brief description of the drawings

Figure 1: RP-HPLC elution profiles of chlorobutyl stopper extract (1:4 diluted) and JEV09L37 SN.

Figure 2: SEC HPLC elution profiles of PS (2mg/mL) before and after trypsin cleavage.

Figure 3: SEC HPLC elution profiles of Trypsin treated PS and degraded PS as present in NIV11A74. Note that elution profiles were normalized to similar peak height to allow better comparison.

Figure 4: DOE evaluation of the ratio monoclonal/polyclonal ELISA by Pareto chart analysis and main effects plots (4 weeks at 22°C).

Figure 5: DOE evaluation of the ratio monoclonal/polyclonal ELISA by Pareto chart analysis and main effects plots (8 weeks at 22°C).

Figure 6: Contour plot of the estimated response

Figure 7: Residual plot of the estimated response

Figure 8: ELISA ratio (monoclonal/polyclonal) for JEV formulations at pH 7 in presence of Ni, Cu and Cr. Samples were stored at 22°C for 5 weeks

Figure 9: Summary of results obtained after 7 weeks at 22°C. Shown are the raw data of ratio as a function of pH and metal ion type and combined results for each parameter.

Figure 10: Mean ratio of DP formulations prepared with different Alum lots. Samples were stored for 6 weeks at 22°C. Error bars represent the 95% confidence interval calculated based on pooled standard deviation. Samples from left to right: Alum 3877, Alum 4074, Alum 4230 nonGI, Alum 4230 GI, Alum 4470, Alum 4563, Alum 4621, Alum Mix 4074_4230.

Figure 11: Particle size distribution of Alhydrogel® samples.

Figure 12: Alhydrogel® titration curves in PBS. ■ non-irradiated AlOH (RQCS0890), ▲ GI AlOH (RQCS1200), ♦ GI AlOH (RQCS1342), + GI AlOH (RQCS0448)

Figure 13: Overview of tested Alhydrogel® batches. Total concentration of contaminating metal ions in ng/mL and share of the main metal ions Fe, Cr and Ni are shown.

Figure 14: Comparison of metal ion contamination in analyzed Ammonia Alum raw material batches; lots 6-80578-28, AA0427 and 91480 were used in JEV vaccine production.

Figure 15: Amino acid sequence of Ala-(His)6-OprF190-342 -OprI21-83 (SEQ ID NO: 1) – herein also referred as “protein A”.

Examples

Example 1

Aluminium hydroxide (Alum) Lot 4230 was previously identified to contribute significantly to antigen degradation in FVL09L37. In this particular Alum lot much higher residual metal ion content was observed compared to other Alum lots used for formulation of the inactivated JEV antigen. This Example demonstrates additional studies carried out to further identify the underlying root-cause mechanism and influence of metal ions on degradation pathway of JEV. Design of experiments (DOE) was performed to work out the influence of individual parameters on antigen stability.

Parameters tested in a 25 full factorial DOE were

- Aluminium hydroxide Lot 4230 vs. Aluminium hydroxide Lot 4074
- Presence of excess Protamine sulfate fragments
- Presence of leachables from chlorobutyl rubber stopper
- pH range 7 to 8
- Residual formaldehyde content

Alum lot 4230 contains much higher levels of residual metal ion impurities compared to other Alum lots used for formulation of JEV. A "Design-of-Experiment" (DOE) was selected to further investigate the potential root cause mechanism and interaction of parameters that finally could lead to product degradation. In factorial designs, multiple factors are investigated simultaneously during the test. As in one factor designs, qualitative and/or quantitative factors can be considered. The objective of these designs is to identify the factors that have a significant effect on the response, as well as investigate the effect of interactions (depending on the experiment design used). Predictions can also be performed when quantitative factors are present, but care must be taken since certain designs are very limited in the

choice of the predictive model. For information about DOE in general see (Siebertz, Karl; van Bebber, David, Hochkirchen, Thomas: Statistische Versuchsplanung: Design of Experiments (DoE). Publisher: Springer Berlin Heidelberg; 1st Edition (2010), ISBN-10: 3642054927).

1.1 DOE Study Design

1.1.1 Definition of Parameters and levels for DOE Design

The following parameters and levels were taken into consideration for designing an appropriate DOE experiment:

- **Residual metal ion content of Alum:** Aluminium hydroxide Lot 4230 and Lot 4074 were selected as representative of the two extremes quality with regard to residual metal ions content of Aluminium hydroxide. The center point was a mixture of 50/50% of both Alum lots. Initial analysis for remaining metal ion impurities in 2% Aluminium hydroxide stock solution by ICP-MS showed significant differences in Cr, Fe, Ni and Cu ion content between these two lots (see Table 1).
- **Protamine Sulphate fragments:** Protamine sulfate (PS) fragments are **present** at low quantity (<5µg/mL) in the final vaccine lot. It was tested if PS fragments could contribute to virus surface modification (e.g. interaction/ covalent linkage to the virus surface proteins) in combination with Alum and other factors used in this study. Therefore a stock solution of PS fragments was prepared by digestion with Trypsin followed by heat inactivation and ultrafiltration using a 5kDa membrane for protease inactivation and removal of the enzyme. This stock solution was used for spiking additional PS fragments into the respective formulations at the high level of 50 µg/mL. In low level samples no additional PS fragments were spiked and the actual level in formulations was < 5µg/mL according to HPLC analysis.
- **pH:** Lower and upper level of pH in formulations was 7 and 8 with the center point at pH 7.5.

- **Leachables/Extractables from syringe plunger**: Syringe plunger (made of chlorobutyl PH701/50 black) that are currently used in the container closure system. It was tested if leachables from the chlorobutyl rubber in the formulation could contribute to antigen modification. Therefore a stock solution of leachables was prepared and used for spiking experiments. The high level of spiked leachables in formulation was estimated to be on average 1.4 x higher compared to commercial Final Vaccine Lot (FVL). Due to the harsh extraction conditions additional peaks were detected not present in FVL samples. Therefore the spiked formulations represent a "worst case" with regard to leachables and extractables. Formulations at the low level did not contain any leachables from chlorobutyl rubber.
- **Residual formaldehyde**: For low level formulations no additional formaldehyde was spiked into the formulation samples. The lower level was the residual formaldehyde that was still present in diluted NIV sample after inactivation/neutralization and 2-fold dilution was in the range of approx. 37 ppm (recalculated from commercial DS release GMP analytical certificate). For the high level additional 40 ppm formalehyde were spiked into the corresponding formulation (total final content approx. 77 ppm). It was tested if residual formaldehyde in combination with higher level of metal ions present in Alum 4230 and possible other factors could further react with the virus leading to hyper-cross linking of surface proteins and loss of relevant epitopes.

Determination of other residual process related impurities:

Residual formaldehyde, sulphite and sucrose in final formulations were estimated based on GMP certificates for commercial drug substance JEV11A74. Results were recalculated by the actual 2-fold dilution of NIV to DS used within the DOE experiments.

Residual Sulphite: The concentration of residual sulphite was constant in all formulations with approx. 93 ppm.

Residual sucrose: The concentration of residual sucrose was constant in all formulations with approx. 1% v/w.

1.1.2 DOE Design

These 5 factors were combined in a 25 DOE plan resulting in a total number of 34 experiments including 2 center points with the following base design. DOE planning and evaluation were carried out with an appropriate software (Statgraphic Plus 3.0)

Base Design: Factorial 25

Number of experimental factors: 5

Number of blocks: 1

Number of responses: 1

Number of centerpoints per block: 2

Number of runs: 34

Error degrees of freedom: 18

Randomized: Yes

Factors	**Low**	**High**	**Units**	
Continuous[1)]				
pH	7.0	8.0		
Yes				
Alum[2)]	0.0	100.0	Alum 4230 %	
Yes				
Spiked PS Fragments[3]	0	50	µg/mL	
No				
Spiked leachables [4)]	0	1.4	relative content compared to FVL	No
Spiked Formaldehyde[5)]	0	40	ppm	
No				

Responses	**Units**
ELISA (monoclonal, polyclonal) of desorbed antigen	AU/mL

1) Continuous means that a center point (mean value from high and low level) is present in the study design. No Center point means that only low and high levels are present in the study design.

2) Low level (0%) means that formulation was prepared with Alum Lot 4074. High level (100%) means that formulation was prepared with Alum lot 4230. For center point formulations an equal mixture (50/50%) of both Alum lots was used.

3) Since PS is present in NIV used for preparation of drug product samples, actual PS concentration in non-spiked formulations was <5µg/mL and ~50-55µg/mL for PS-spiked formulations.

4) No leachables were assumed in non-spiked formulations since samples were prepared/stored in low-bind Eppendorf tubes. Total content of leachables from chlorobutyl rubber syringe plunger in spiked samples was approx. 1.4 times higher compared to FVL.

5) Actual formaldehyde concentration in non-spiked DP samples was approx. 37ppm, total formaldehyde concentration in spiked samples was approx.77ppm.

2 Definitions & Abbreviations

AcCN Acetonitrile

DOE Design of experiments

DS Drug substance

FVL Final Vaccine lot

HPLC High performance liquid chromatography

ICP-MS Inductively-coupled-plasma mass spectrometry

PS Protamine sulphate

RP Reversed Phase

SEC Size exclusion chromatography

SN Supernatant

TFA Trifluoroacetic acid

w/o without

3 Materials and Methods

3.1 DOE Studies

3.1.1 Materials

- Syringe plunger stoppers: PH701/50/C black Sil6 7002-1051 (obtained from West, Order No. 2116)
- 100 mL Glass bottle (Schott) + Teflon coated screw cap
- Aluminium foil
- HQ water
- Electrical water bath (IKA, HBR 4 digital)
- LoBind Eppis 2ml (Eppendorf, Cat. No. 0030 108.132)
- Speed Vac (Christ, RVC-2-25)
- HPLC vials, clear glass, 900 µL, Chromacol (VWR, Cat.no.: 548-1124)
- HPLC vials, PP, 900 µL, (Agilent, Item no. 5182-0567)
- Caps for HPLC vials, pre-cut (VWR, Cat.no.: 548-1260)
- 15ml Falcon tubes (Greiner, Cat. No. 188724)
- Alum batch 4470 (RQCS 1342); Alum Lot 4230 (RQCS 1200)
- 10xPBS (Gibco, Order No. 14200-091)
- Parafilm
- Waters Atlantis T3 column; 3µm particle diameter; column diameter/length 2.1x100mm (Order No. 186003718; Lot 0107372331)
- Acetonitril (Merck, Cat No. 1.13358.2500)
- TFA (Sigma, Order No. 302031
- HPLC system Dionex 3000
- Solvent Rack SR-3000
- Pump UltiMate-3000, analytical low pressure gradient pump
- Autosampler WPS-3000 TSL, analytical autosampler - temperature controlled
- Column compartment TCC-3200, temperature controlled
- PDA-Detector PDA-3000

- Formaldehyde solution 37% (Merck, Cat No. 1.040031000)
- Protamine sulphate (Intercell Biomedical Ltd, Batch no. 086056)
- Ultrafilatrion device (Amicon® Ultra 3 kDa) (Millipore, Cat No. UFC900324)
- Incubator Infors HT Incubator Multitron Standard (InforsAG)
- Trypsin (Sigma, Order No: T0303)

3.1.2 Procedure for preparation of extractables from syringe plunger

Syringe plunger (made of chlorobutyl PH701/50 black) that are currently used in the FVL container closure system were obtained from West (Germany). Therefore a stock solution of leachables was prepared by heat treatment of syringe plunger in water (90°C/2h) followed by concentration in a speed-vac. The relative content of leachables in this stock solution was estimated by RP-HPLC using a C18 column (Atlantis T3 column) and compared to FVL JEV09L37 supernatant.

Extraction method

A 100 mL Schott glass bottle with a Teflon coated screw top and a piece of aluminum foil were washed with hot water and thoroughly rinsed with HQ water. 30 stoppers were filled in the bottle and 30ml of HQ-water were added. The bottle was closed with the aluminum foil fitted between bottle and screw and sealed additionally with Parafilm. The bottle was heated in the water bath to 90°C for 2 hours and allowed to cool to room temperature. The extract was transferred to 14 low-bind Eppendorf tubes (a total of 28 mL extract was recovered). Twelve vials (total of 24 mL) were concentrated in a Speed Vac for approximately 44 hours and pooled into a falcon tube to obtain 6 ml of 4x concentrated stopper extract. A control sample containing 30 mL HQ water w/o stopper was prepared in the same way to evaluate any possible contamination.

C18 RP-HPLC method

Leachables were separated by RP-HPLC C18 column (Atlantis T3) operated at 40°C and 0.25 mL/min. Solvent A was 0.1% TFA in H2O, solvent B was 0.1% TFA in AcCN. Separation was performed by linear gradient ranging from 0 to 95% B in 30 min. Detection was done at 214 nm, 254 nm and 280 nm. The total relative concentration of concentrated stopper extract was estimated to be 80 fold higher compared to peaks detected in Final Vaccine Lot supernatant (FVL SN; obtained by removal of Alum particles by centrifugation at 5000g/5min) as detected at 254 nm. Therefore a total relative content of 80 U/mL (arbitrary Units U) were assigned for the stock solution, whereas the total relative concentration of leachables in FVL SN was set to 1U/mL. For DOE studies, the stock solution was diluted 16-fold into the respective formulations yielding approx. 5 U/mL of total extractables.

3.1.3 Preparation of Protamine Sulfate fragments

A stock solution of PS fragments was prepared by digesting a PS solution (2mg/mL in PBS) with Trypsin (200 ng/mL for 60 min at 37 °C). The enzyme was subsequently inactivated by heat (90°C for 10 min) followed by ultrafiltration using a 3kDa membrane (Amicon® Ultra centrifugal filter). Due to the cut-off of the membrane Trypsin remained in the retentate, whereas the PS fragments were present in the permeate. Complete inactivation of the enzyme was evaluated by spiking 500 µg/mL of full length PS into an aliquot of the obtained PS fragment followed by incubation at 37°C for 18 h. No degradation of full length PS was observed indication complete inactivation/removal of Trypsin. Degradation was monitored by PS-SEC HPLC.

3.1.4 DOE Plan

Samples were prepared according to the pipetting scheme as shown in Table 2. NIV Batch JEV11A74 obtained from a commercial production run was used as

starting sample. NIV was diluted 2-fold to DS using PBS buffer followed by pH adjustment. 5 mL aliquots were removed and adjuvanted with the corresponding Alum lot 4230, 4074 or a 50/50% mixture of both. The final amount of Alum stock (2% Al2O3) added was 500µg/mL Aluminium (0.1% Al2O3). Each formulation (5 mL) was split into two parts (2 x 2.5 mL) using Lo-bind Eppendorf tubes. One aliquot was stored at 2-8°C, another aliquot stored at 22 ± 1°C (Infors HT Incubator) under gentle shaking (20 rpm).

3.2 Inactivated JEV ELISA (polyclonal based)

Desorption of the antigen from Alum and ELISA analysis was carried out using polyclonal sheep anti JEV antibodies for coating the 96 well ELISA plates as described in Example 4.

3.3 Inactivated JEV ELISA (monoclonal based)

A monoclonal (mAb) based JEV ELISA was developed. The assay is primarily based on the "polyclonal JEV ELISA" assay format, only a monoclonal anti-JEV antibody (clone 52-2-5) is used for coating. The employed mab 52-2-5 was shown to be specific for JEV and to recognize a neutralizing epitope. Mab clone 52-2-5 was obtained by subcutaneously immunizing BALB/c mice with commercially available vaccine lot JEV08J14B. Spleen cells of the mice were fused to myeloma cells. From resulting hybridoma cells single clones were selected and sub-cloned. The clones were negatively screened against Bovine Serum Albumin, Protamine sulphate and an extract of the production cell line of the JE-vaccine (Vero cells). A positive screen was done against Neutralized Inactivated Virus (NIV) of vaccine lot JEV08M20. For screening, microtiter plates were coated with the relevant antigen and reacted with supernatant of cultures of the selected clones. For detection a goat anti mouse polyclonal antibody conjugated with alkaline phosphatase was used. Mab clone 52-2-5 was shown to recognize a neutralizing epitope on domain III of the envelope (E) protein of JEV containing Ser331 and Asp332 (Lin C.-W. and Wu W.-C. J Virol. 2003 ;77(4):2600-6). Binding of the mab to the indicated neutralizing

epitope is for instance determined as described in Lin and Wu (2003) by site-directed mutagenesis of the domain III at position 331 (for instance: S→R), and/or by alanine mutations at or near position 331 of domain III, for instance of residues Ser 331 and Asp332, followed by immunoblots to determine binding of the mab to the mutated proteins. Negative binding results indicate that the epitope of the mab is the neutralizing epitope identified by Lin and Wu (2003). The neutralizing characteristic of the epitope gives rise to the assumption that the epitope might be of importance for the antigen to elicit a protective immune response.

JEV samples were analyzed by both ELISA assays, polyclonal and monoclonal. The relative specific epitope content can be expressed as the ratio of the total antigen content determined by "monoclonal ELISA" (clone 52-2-5) divided by total antigen content determined by "polyclonal ELISA". Any differences in the ratio may indicate differences in specific epitope content 52-2-5. Results close to 1 would correspond to high epitope contents, and results close to 0 correspond to low relative epitope content. A low ratio indicates presence of structural changes of the neutralizing epitope.

In the course of development of this "mAb ELISA", differences between vaccines lots were detected, which could be correlated with potency results of these lots.

3.4 Protamine sulfate SEC-HPLC

PS (full length) and its fragments were analyzed by size-exclusion HPLC (SEC-HPLC) using a Superdex Peptide 10/300 GL, 10 x 300 mm, 13 µm (GE Healthcare) using 0.1 % (v/v) Trifluoroacetic acid (TFA) in 30 % acetinitrile (CAN) as mobile phase at a flow rate of 0.6 mL/min. PS containing samples were prepared in duplicated, i.e. diluted with mobile phase before injection.

4 Results

4.1 Analysis of Stopper Leachables used for spiking Experiments

RP-HPLC elution profiles of concentrated stock solution obtained after extraction of stoppers under heat compared to FVL SN is shown in Figure 1. Similar peak pattern as observed for both samples. Due to the harsh extraction conditions additional peaks were detected in the concentrate that were not present in FVL samples or present only at a much lower relative content Therefore the spiked formulations represent a "worst case" with regard to leachables and extractables. The total relative content of individual peaks in extract concentrate and spiked formulation in comparison to FVL SN is summarized in Table 3. The total amount of leachables in the stock solution was calculated as the sum of all peaks detected and expressed in arbitrary units as 67 U/mL. Since the stock solution was diluted 16 times into the respective formulation, the resulting total content of leachables was estimated as 4.2 U/mL. This corresponds on average 1.4 fold increase compared to FVL JEV09L37 supernatant (3.0 U/mL).

4.2 Analysis of Protamine sulphate fragments

PS fragments obtained after cleavage of full length PS by Trypsin are shown in Figure 2. Similar peak profiles of Trypsin treated PS and already degraded PS present in NIV11A74 were obtained by HPLC (see Figure 3).

4.3 DOE Evaluation

Formulations prepared for this DOE were analyzed after 4 weeks and 8 weeks of incubation at accelerated conditions (22°C). It was considered that any degradation reaction would be accelerated when stored at higher temperature compared to normal storage conditions (2-8°C). However, samples are still stored at 2-8°C and will be analyzed on a later time point (~4-6 month). First analysis of samples stored at 22°C for 4 and 8 weeks are shown in the Table 4.

4.3.1 DOE Evaluation after 4 weeks at 22°C

Statistical evaluation of the DOE matrix results obtained after 4 weeks at 22°C showed that the specific epitope content 52-2-5 (expressed as the ratio of desorbed antigen analyzed by monoclonal/polyclonal ELSIA) was statistically significant influenced (95% confidence level, see Table 5) by the following factors:

- lower specific epitope content 52-2-5 in presence of Alum lot 4230
- lower specific epitope content 52-2-5 at lower pH 7
- Higher specific epitope content 52-2-5 at increased concentration of formaldehyde

Presence of higher concentration of PS fragments and chlorobutyl rubber leachables did not show any influence on specific epitope content. No 2nd or higher order interactions between individual parameters were detected.

The ANOVA table partitions the variability in "Ratio 4 weeks" into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 3 effects (Alum, pH, Formaldehyde) have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 74.85% of the variability in Ratio 4 weeks. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 51.27%. The standard error of the estimate shows the standard deviation of the residuals to be 0.063. The mean absolute error (MAE) of 0.0353 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the DW value is greater than 1.4, there is probably not any serious autocorrelation in the residuals. Effects are also displayed by standardized Pareto chart and main effect plots as shown in Figure 4.

4.3.2 DOE Evaluation after 8 weeks at 22°C

Statistical evaluation of the DOE matrix results obtained after 8 weeks at 22°C were similar to results obtained after 4 weeks. Evaluation shows that the specific epitope content 52-2-5 (expressed as the ratio of desorbed antigen analyzed by monoclonal/polyclonal ELSIA) was statistically significant influenced (95% confidence level, see Table 6) by the following factors:

- lower specific epitope content 52-2-5 in presence of Alum lot 4230
- lower specific epitope content 52-2-5 at lower pH 7

Presence of higher concentration of PS fragments, chlorobutyl rubber leachables and formaldehyde did not show any influence on specific epitope content. Note that P-value for formaldehyde (P=0.08) is quite close to be significant. No 2nd or higher order interactions between individual parameters were detected.

The ANOVA table partitions the variability in "Ratio 8 weeks" into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 2 effects (Alum and pH) have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 75.9% of the variability in "Ratio 8 weeks". The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 53.3%. The standard error of the estimate shows the standard deviation of the residuals to be 0.095. The mean absolute error (MAE) of 0.057 is the average value of the residuals. Effects are also displayed by standardized Pareto chart and main effect plots as shown in Figure 5.

Regression analysis was also performed to the fitted data and calculated regression coefficients are shown in Table 7. The regression equation is

displayed below which has been fitted to the data including pH, Alum and Formaldehyde. The equation of the fitted model is

"Ratio 8 weeks" = 0.0228125 + 0.113125*pH - 0.00185625*Alum +0.0315625*Formaldehyde

where the values of the variables are specified in their original units, except for the categorical factors which take the values -1 for the low level and +1 for the high level. The contour of the estimated response and residual plot is shown in Figure 6 and Figure 7. The ratio increases when relative Alum content Lot 4230 decreases and pH increases.

Table 8 contains information about values of "Ratio 8 weeks" generated using the fitted model. The table includes:

(1) the observed value of "Ratio 8 weeks"

(2) the predicted value of "Ratio 8 weeks" using the fitted model

(3) 95.0% confidence limits for the mean response

As shown the experimental results are well predicted by the regression model.

5. Summary

Out of the parameters tested, Alum lot 4230 was shown to contribute significantly to antigen degradation as analyzed by monoclonal/polyclonal ELISA under accelerated conditions (22°C, testing time points 4 and 8 weeks). DOE results obtained after 4 and 8 weeks at 22°C show that Alum 4230 is the most significant factor with regard to antigen degradation as detected by the ratio of monoclonal/polyclonal ELISA. Formulations made with Alum 4074 (much higher purity with regard to residual metal ions) show in general much higher specific epitope content.

Formalaldehyde and pH also contributed to antigen stability, but to a lower extent. The effect of increased antigen stability in samples formulated with Alum 4230 at higher formaldehyde level was well demonstrated (e.g. samples

#19 and 29). However, influence of formaldehyde was less pronounced after extended storage period (8 weeks at 22°C).

Better stability of the antigen was observed at pH 8 compared to pH 7.

Protamine sulphate and leachables from chlorobutyl rubber stopper did not contribute to the antigen degradation.

Example 2

In previous studies (see Example 1) Aluminium hydroxide Lot 4230 was identified a significant contributing factor to the observed antigen degradation in FVL09L37. In this particular lot of Aluminium hydroxide (Alum), a much higher residual metal ion content was observed compared to other Alum lots used for formulation of the inactivated JEV antigen. This Example summarizes additional studies carried out to evaluate the influence of metal ions on stability of inactivated JEV. Spiking studies were conducted with the antigen either present in inactivated neutralized virus (NIV) solution or in drug product (DP) suspension following formulation of the antigen with Aluminium hydroxide.

1 Study Description

It was previously shown that Alum lot 4230 contains much higher levels of residual metal ion impurities compared to other Alum lots used for formulation of JEV (see also Example 3). Additional studies were performed to evaluate the influence of metal ions on the stability and on a potential surface modification of JEV. The inactivated antigen was either present in neutralized inactivated virus (NIV) solution or in drug product (DP) suspension following further dilution of NIV and formulation with Aluminium hydroxide. In another set of experiments, different Alum lots covering a broad content range of residual metal ions were used and formulated with a single defined NIV lot. All of these formulations still contained residual formaldehyde and bisulphite at representative concentrations compared to commercial product. Stock solution of metal salts were dissolved in water and spiked to the samples to the desired final concentration.

2 Definitions & Abbreviations

AcCN Acetonitrile

ANOVA Analysis of variance

DOE Design of experiments

DP Drug product

DS Drug substance

FBV Final bulk vaccine

FVL Final Vaccine lot

GI Gamma irradiated

HPLC High performance liquid chromatography

LSD Fisher's least significant difference

mAb Monoclonal antibody

NIV Neutralized inactivated virus

PS Protamine sulphate

RP Reversed Phase

SEC Size exclusion chromatography

SN Supernatant

TFA Trifluoroacetic acid

w/o without

3 Materials and Methods

3.1 Materials

- Iron(II)chloride tetrahydrate(Sigma, Order no. 44939)
- Iron(III)chloride hexahydrate (Sigma, Order no. 31232)
- Nickle(II)sulphate hexahydrate (Sigma, Order no. N4882)
- Cobalt(II)chloride hexahydrate (Sigma, Order no. 31277)
- Copper(II)chloride dehydrate (Sigma, Order no. 807483)
- Zinksulphate heptahydrate (Sigma, Order no. 24750)
- Crom(III)chloride hexahydrate (AlfaAesar, Order no. 42114)

- Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (Sigma, E5134)
- Aqua bidest. (Fresenius Kabi, Art no. 0712221/01 A)
- 10xPBS (Gibco, Order No. 14200-091)
- Formaldehyde solution 37% (Merck, Cat No. 1.040031000)
- Protamine sulphate (Intercell Biomedical Ltd, Batch no. 086056)
- LoBind Eppis 2 ml (Eppendorf, Cat. No. 0030 108.132)
- 15 ml Falcon tubes (Greiner, Cat. No. 188724)
- Incubator Infors HT Incubator Multitron Standard (InforsAG)
- 0.2 µm filter Mini Kleenpak 25 mm (Pall)
- NIV11A74 and Final bulk vaccine (FBV, formulated with Alum lot 4539) JEV 11D87 from commercial production runs was obtained from Intercell Biomedical (Livingston, UK) and stored at 2-8°C until further processing
- Stock solutions of metal salts in water (final concentration 1mM) used for spiking experiments were prepared and stored at 2-8°C until usage
- Aluminium hydroxide samples (2% Al2O3, Brenntag Biosector) were either retain samples obtained from Intercell Biomedical or purchased directly from Brenntag. Alum samples were stored at 2-8°C. The following Alum lots were used in this study: 4470, 4563, 4621, 3877, 4230 (non gamma irradiated and gamma irradiated)

3.2 Preparation of metal stock solutions

3.2.1 Iron(II) stock solution

20 mM Iron(II) stock solution was prepared by dissolving 397 mg of Iron(II)chloride tetrahydrate in 100 mL aqua bidest.

3.2.2 Iron(III) stock solution

20 mM Iron(III) stock solution was prepared by dissolving 540 mg of Iron(III)chloride hexahydrate in 100 mL of aqua bidest.

3.2.3 Nickle(II) stock solution

20 mM Nickle(II) stock solution was prepared by dissolving 525 mg of Nickle(II)sulphate hexahydrate in 100 mL of aqua bidest.

3.2.4 Cobalt(II) stock solution

20 mM Cobalt(II) stock solution was prepared by dissolving 476 mg of Cobalt(II)chloride hexahydrate in 100 mL of aqua bidest.

3.2.5 Copper(II) stock solution

20 mM Copper(II) stock solution was prepared by dissolving 341 mg of Copper(II)chloride dihydrate in 100 mL of aqua bidest.

3.2.6 Zink stock solution

20 mM Zink stock solution was prepared by dissolving 575 mg of Zinksulphate heptahydrate in 100 mL of aqua bidest.

3.2.7 Crom(III) stock solution

20 mM Crom(III) stock solution was prepared by dissolving 533 mg of Crom(III)chloride hexahydrate in 100 mL of aqua bidest.

3.3 Preparation of working solutions

Working solutions of metal ions (1 mM final concentration if not otherwise stated) were prepared by dilution of metal ion stock solutions with aqua bidest and sterile filtration via 0.2 µm syringe filter.

3.4 Preparation of Formulation

All formulations were prepared under sterile conditions. NIV and FBV obtained from commercial production runs were adjusted to the desired pH and spiked with aliquots of metal stock solution. All samples were stored in

plastic tubes if not otherwise stated. In all formulations using Alum, the final Al content was 500µg/mL, corresponding to 0.1% Al2O3. It has to be noted that metal ions, especially iron (II), iron (III) and to a certain extent Cr (III), formed a precipitate with the phosphate ions present in the buffer resulting in partial co-precipitation of the inactivated virus represented by the low recovery determined by size-exclusion HPLC (SEC-HPLC).

3.4.1 Experiment 20110913(NIV): NIV Formulation at different metal ion concentration of Ni(II), Cu(II), Cr(III) with or w/o presence of PS fragments

NIV 11A74 was adjusted to pH 7 and pH 8 followed by spiking of metal ions (Ni(II), Cu(II), Cr(III)) at 100/500/1000 ng/mL final concentration. All formulations were stored in low-bind Eppendorf tubes at 22°C. Aliquots of all formulation were also prepared in presence of protamine sulphate fragments (50µg/mL). This was done to evaluate for any effect of PS fragments on JEV stability in presence of metals. The preparation of Protamine Sulphate (PS) fragments is described in Example 1. Samples were prepared on the same day (see Table 9) and analyzed three weeks later. All samples were analyzed by SEC-HPLC, but only samples at pH 8 (#21-40) were analyzed by ELISA.

3.4.2 Experiment 20110913(DP): DP Formulation at different metal ion concentration of Ni(II), Cu(II), Cr(III)

FBV 11D87 (formulated with Alum Lot 4539) was used in this study. FBV was adjusted to pH 7 and pH 8 and spiked with Ni(II)/Cu(II)/Cr(III) at 100, 500 and 1000ng/mL to evaluate any metal ion concentration /pH depended effect. Table 10 shows the experimental design of this experiment. All formulations were stored in Falcon tubes at 2-8°C and 22°C. Samples stored at 22°C were analyzed by SEC-HPLC and ELISA after 5 weeks.

3.4.3 Experiment 20110812-Metal Spiked DP

Final Bulk Vaccine 11D87 (formulated with Alum Lot 4539) was obtained from a commercial production run and used in this study. Residual formalin in DS was analyzed as 28.1ppm, residual sulphites was 92.2ppm. Actual content in DP can be considered to be in the same range. FBV JEV11D87 was adjusted to pH 7.0/7.4/7.8 and spiked with 500ng/mL (final concentration) of Fe(II), Fe(III), Ni(II), Co(II), Cu(II), Zn(II). A metal ion mix formulation was also prepared containing all of the individual metal ions together in solution. Formulations with Cr(III) were prepared later on and Cr(III) was not included in metal ion mix. Control formulations were only adjusted to the desired pH, but not spiked with metals. All formulations (#1-24) were prepared on the same day and stored in Falcon tubes at 2-8°C and 22°C.
Additional Cr(III) spiked samples (#25-27) were prepared by taking aliquots of the control samples stored at 2-8°C and spiked with Cr(III) to a final concentration of 500ng/mL. Formulations were stored at accelerated conditions (22°C) only. Table 11 shows the experimental set-up of this experiment. All samples stored at 22°C were analyzed by ELISA (monoclonal and polyclonal) after 4 weeks and 7 weeks.

3.4.4 Experiment 20110819: DP Formulation using various Alum lots

Spiking studies as described above can give first evidence of possible instability of the formulated antigen in presence of certain metals, but might not be completely representative of the real conditions where metals present in Aluminium hydroxide are incorporated in the three-dimensional structure of the gel resulting in different local concentration and orientation/accessibility. To overcome these limitations an initial study was started to simulate the real conditions. A single NIV batch (11A74) obtained from a commercial production run was formulated with various Alum lots produced by Brenntag covering a

broad range of residual metals. 4.75mL of NIV was mixed with 0.25mL Alum (2%) in Falcon tubes. The final Aluminium hydroxide concentration was 500µg/mL (=0.1% Al2O3). Formulated vaccine samples were stored at 2-8°C and under accelerated conditions at 22°C. All of these Alum lots contained residual metal ions at different concentrations. Alum lot 4230 has the highest level for Fe, Cu, Ni and V (see Example 3). Note that metal ion valences cannot be specified by ICP-MS. A mixed Alum sample containing equal amounts of 4230 and 4074 was also prepared to get an "intermediate" level for Ni(II) and Cu(II). Samples were analyzed after 6 weeks of storage at 22°C. The residual amount of formaldehyde and sulphite estimated by recalculation from available DS analysis results corrected by dilution factor of NIV to DS was 76ppm formaldehyde and 192ppm sulphite respectively.

3.1 Antigen Desorption from Aluminium Hydroxide for SEC-MALLS analysis

Viral particles were desorbed from Aluminium hydroxide. ~625 µL of DP was spun down (8°C, 5 min, 3300 x g) and the supernatant was either discarded if not otherwise stated or analyzed by JEV-SEC-MALLS to detect the unbound antigen concentration. Viral particles were desorbed by suspending the Aluminium hydroxide particles with 62.5 µL 0.8 M potassium phosphate buffer (pH 8) containing BSA (50 µg/mL). BSA was added to the desorption buffer for SEC-MALLS analysis to minimize losses caused by unspecific adsorption of the antigen. After shaking (500 rpm) the Aluminium hydroxide particles for 10 min at room temperature, particles were removed by centrifugation and the supernatant was collected into a LoBind Eppendorf tube and the desorption procedure repeated on remaining sample. The pooled desorbed antigen (~5× concentrated sample; final volume 125µL; starting volume ~625 µL) was then further analyzed by SEC-MALLS.

3.2 SEC-MALLS HPLC Method

Desorbed antigen was analyzed by SEC-MALLS. In brief, following desorption of the antigen from Aluminium hydroxide 100 µL of the pooled desorbed material (~5× concentrated) were subsequently loaded onto a Superose 6 10/300 GL SEC column. 1× PBS + 250 mM NaCl was used as mobile phase. Ultraviolet (UV) 214 nm and MALLS signals of viral particles were recorded and analysed using Chromeleon and ASTRA software packages.

3.3 Inactivated JEV ELISA (polyclonal based)

Desorption of the antigen from Alum and ELISA analysis was carried out using polyclonal sheep anti JEV antibodies for coating the 96 well ELISA plates as described in Example 4.

3.4 Inactivated JEV ELISA (monoclonal based)

During course of this investigational testing, a monoclonal (mAb) based JEV ELISA was developed. The assay is primarily based on the "polyclonal JEV ELISA" assay format, only a monoclonal anti-JEV antibody (clone 52-2-5) is used for coating and the current polyclonal antibody for detection. The employed mab 52-2-5 was shown to be specific for JEV and to recognize a neutralizing epitope. Mab clone 52-2-5 was obtained by subcutaneously immunizing BALB/c mice with commercially available vaccine lot JEV08J14B. Spleen cells of the mice were fused to myeloma cells. From resulting hybridoma cells single clones were selected and sub-cloned. The clones were negatively screened against Bovine Serum Albumin, Protamine sulphate and an extract of the production cell line of the JE-vaccine (Vero cells). A positive screen was done against Neutralized Inactivated Virus (NIV) of vaccine lot JEV08M20. For screening, microtiter plates were coated with the relevant antigen and reacted with supernatant of cultures of the selected clones. For detection a goat anti mouse polyclonal antibody conjugated with alkaline phosphatase was used. Mab clone 52-2-5 was shown to recognize a neutralizing

epitope on domain III of the envelope (E) protein of JEV containing Ser331 and Asp332 (Lin C.-W. and Wu W.-C. J Virol. 2003 ;77(4):2600-6). Binding of the mab to the indicated neutralizing epitope is for instance determined as described in Lin and Wu (2003) by site-directed mutagenesis of the domain III at position 331 (for instance: S→R), and/or by alanine mutations at or near position 331 of domain III, for instance of residues Ser 331 and Asp332, followed by immunoblots to determine binding of the mab to the mutated proteins. Negative binding results indicate that the epitope of the mab is the neutralizing epitope identified by Lin and Wu (2003). The neutralizing characteristic of the epitope gives rise to the assumption that the epitope might be of importance for the antigen to elicit a protective immune response. JEV samples were analyzed by both ELISA assays, polyclonal and monoclonal. The relative specific epitope content can be expressed as the ratio of the total antigen content determined by "monoclonal ELISA" (clone 52-2-5) divided by total antigen content determined by "polyclonal ELISA". Any differences in the ratio may indicate differences in specific epitope content 52-2-5. Results close to 1 would correspond to high epitope contents, and results close to 0 correspond to low relative epitope content. A low ratio indicates presence of structural changes of the neutralizing epitope.

In the course of development of this "mAb ELISA", differences between vaccines lots were detected, which could be correlated with potency results of these lots.

3.5 Statistical Evaluation

Statistical evaluation was done with Statgraphic Plus 3.0.

4 Results

4.1 Experiment 20110913(NIV): NIV Formulation at different metal ion concentration of Ni(II), Cu(II), Cr(III) with or w/o presence of PS fragments

SEC-HPLC results of NIV formulations (pH 7 and pH 8) containing metal ions [Ni(II), Cu(II), Cr(III)] w/ and w/o PS fragments are summarized in Table 12. SEC-HPLC results show that antigen recoveries of most of the samples was >80%. Some samples (#7, #36, #38) showed slightly reduced recoveries in the range of 70-80%. It has to be noted that the actual virus content is quite low and precision of HPLC results can be estimated as approx.± 20%. Since for samples #36 and #38 the recoveries for following formulations (#37, #39) at next level of individual metal ion content were higher again, these differences might be caused by assay variability and were not considered as significant. Based on the results obtained it was not possible to clarify the influence of metal ions with respect to the recovery of soluble inactivated JEV. However, SEC-HPLC only gives information about content of soluble virus, but no information about any potential surface modification. Only formulations prepared at pH 8 were also analyzed by ELISA (duplicate analysis). The ratio of monoclonal/polyclonal ELISA was calculated and can be used for comparison purpose of results. Analysis of samples by ELISA (see Table 13) do not show any significant influence of tested metals on degradation of inactivated JEV at pH 8 after three weeks at 22°C. There might be a trend of decreasing ratio in presence of Cu(II), but overall it appears that an incubation time of three weeks at 22°C seems not be sufficient to detect any significant degradation. As also shown in DOE experiment (Example 1) inactivated JEV appears to have higher stability at pH 8 when stored at accelerated conditions at 22°C and this would also contribute that significant effects were not observed. In this experiment it was also shown that PS fragments do not have any influence on JEV stability. This is also well in agreement with DOE results. NIV samples 1-

20 formulated at pH 7 showed significant reduction in monoclonal epitope content in presence of Cu(II). At the highest tested concentration (1000ng/mL) the ratio was close to zero indication significant structural changes of the antigen.

5

4.2 Experiment 20110913(DP): DP Formulation with different metal ion concentration of Ni(II), Cu(II), Cr(III)

Analysis of desorbed JEV antigen is summarized in Table 14 (SEC-HPLC) and Table 15 (ELISA). Antigen recoveries for all samples as determined by SEC-
10 HPLC was >80% after 5 weeks at 22°C indicating no significant influence of tested metal ions on desorption recovery. As shown in Figure 8, there is a trend of decreasing ratio as analyzed by ELISA in presence of Cu(II) and Cr(III) at pH 7. Formulations at pH 8 appear to be more stable.

15 **4.3 Experiment 20110812(DP): Metal Ion Spiked DP**

In this experiment FBV11D87 was used as starting material. The formulation pH value was adjusted in a more narrow range (pH 7.0, 7.4 , 7.8) and additional metal ions were used for spiking, each at 500ng/mL (final concentration). The metal ion mix contained all individual metal ions with the
20 exception of Cr(III) in single formulations (each metal at 500ng/mL). ELISA results obtained after 4 weeks and 7 weeks at 22°C are summarized in Table 16. Results are also displayed as graphs in Figure 9.

Statistical evaluation of stability samples stored at 22°C for 7 weeks was
25 performed. ANOVA (analysis of variance) showed significant effects of parameters (pH and metal type) on antigen stability, that is expressed as the ratio of monoclonal/polyclonal ELISA (see Table 17). The ANOVA table decomposes the variability of Ratio into contributions due to various factors. Since Type III sums of squares have been chosen, the contribution of each
30 factor is measured having removed the effects of all other factors. The P-values

test the statistical significance of each of the factors. Since the P-values for pH and metal ion type are less than 0.05, these factors have a statistically significant effect on Ratio at the 95.0% confidence level.

In Table 18 a multiple comparison procedure is applied to determine the significance of the differences observed with respect to the means. Significant effect on ratio was shown for Cu(II) and the metal mix compared to the non-spiked control formulations. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 7 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 3 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Significant effect on ratio was shown for Cu(II) and the metal ion mix. The influence of other metal ions might become significant at longer storage period. The metal ion mix contained the highest total concentration and might represent a worst case. However, it was concluded that several metal ions present in Alum might contribute to degradation, each to different extent. These results further support the proposed root cause of metal ion-catalysed antigen degradation. It has to be noted that spiking experiment might not fully simulate the real conditions of residual metal ion impurities present in Alum 4230. Metal ions are incorporated in the Alum structure and the local concentration and orientation might be different from metal ions used in spiking experiments. It is also known that metal ions (e.g. Fe) have low solubility in presence of phosphate ion (PO43-). Therefore the actual

concentration of soluble metal ions and contribution of metals present as metal-phosphate complex on JEV degradation is unknown.

4.4 Experiment 20110819: Preparation of DP samples with different Alum lots

Spiking studies as described earlier can give first evidence of possible instability of the formulated antigen in the presence of some metal ions, but might not be completely representative of the real conditions where these metal ions present in Aluminium hydroxide are expected to be incorporated in the three-dimensional structure of the Aluminium-hydroxide gel resulting in different local concentration and orientation/accessibility. To overcome these limitations an initial study was started to simulate the real conditions. A single NIV (11A74) was formulated with various Alum lots obtained by Brenntag covering a broad range of residual metals. Formulated vaccine samples were stored at 2-8°C and under accelerated conditions at 22°C. All of these Alum lots contained residual metal ions at different levels. Lot 4230 had the highest level for Fe, Cu, Ni and V (see Table 19). Note that the actual content of metal ions present in the formulated product is only 1/20 of the concentration in Alum (2%) stock solution. Note that metal ion valences cannot be specified by ICP-MS. Analysis of desorbed antigen by ELISA of samples stored at 22°C for 6 weeks is shown in Table 20.

Pooled standard deviation was calculated from all samples (spooled ~ 0.075) as a measurement of experimental uncertainty. Mean values for ratio and 95% confidence intervals (calculated based on spooled) were plotted against the individual formulations (see Figure 10). Samples formulated with Alum 4230 showed a trend to lower ratio compared to the other samples. However, differences were not as large to show statistical significant differences between the various formulations.

5 Summary

It was shown that metal ions contribute to the degradation of the inactivated JEV under accelerated storage conditions (22°). In spiking studies higher concentration of residual metal ions (range 100 -1000 ng/mL) were used than present in FVL formulated with Alum lot 4230 (e.g. Fe~310 ng/mL; Cr~64ng/mL; Ni~52ng/mL). Cu content in FVL can be only estimated as 3ng/mL based on ICP-MS data of 2% Alum stock solution since LOD is 25 ng/mL. Higher metal concentration and storage temperature were chosen to increase the rate of any potential degradation reaction. In fact, for FVL JEV09L37, the potency loss occurred after 11 month stored at 2-8°C. It was also shown that metals can form insoluble complexes with phosphate ions making estimation of actual levels of metals present difficult.

In spiking experiments ELISA results showed statistically significant structural changes of virus surface in as little as 4 weeks at 22°C in presence of metal ions. It was shown that the ELISA ratios for formulations containing Cu(II) and metal ion mix (containing Fe(II), Fe(III), Co(II), Cu(II) and Zn(II)) were statistically significant lower compared to the non-spiked control formulation. Cu(II) was also found in Alum lot 4230 (2% stock solution) at 64ng/mL, corresponding to ~3ng/mL in FVL. In all other Alum (2%) lots Cu(II) content was < 25ng/mL (below limit of detection).

For formulation experiments of the antigen using different Alum lots, longer storage time (>6 weeks at 22°C) at accelerated conditions is required. There is a trend that formulations prepared with Alum 4230 showed lower ELISA ratios compared to other lots. Slower degradation rate compared to spiked formulation might be contributing to lower metal ion content in commercial Alum lots. It was also observed that the antigen shows higher stability at pH 7.5-8 compared to pH 7 and that PS fragments do not contribute to any degradation reaction. These results are in good agreement with DOE results described in Example 1.

Example 3

As part of the out-of-specification investigation concerning FVL JEV09L37, the used Aluminum hydroxide lot (lot 4230) was determined to be the most probable root cause for the observed loss of potency. Aluminum hydroxide (referred to as Alum during the manufacturing process of JE-PIV) is purchased from Brenntag Biosector as autoclaved suspension termed "Alhydrogel® Aluminium Hydroxide Gel Adjuvant". Each batch is sterilized by radiation prior to use in the JEV production process.

A number of different Alhydrogel® batches were analyzed for appearance, metal ion content and physical properties.

1 Introduction

1.1 Aluminum hydroxide

Brenntag Biosector's Alhydrogel® has a specified Aluminum content of 10 mg/mL which translates to 2% Al2O3 and 3% Al(OH)3, respectively. Further specifications are Nitrogen (max 0.005%), free Sulphate (max 0.05%), total Sulphate (max 0.1%) and pH (6.5 ± 0.5). it has a shelf life of 26 months when stored at room temperature.

1.2 Generation of Aluminum hydroxide

Alhydrogel® 2% (referred to as Aluminum hydroxide) is manufactured by Brenntag (CAS no. 21645-51-2).

It is noteworthy, that after the initial precipitation by Ammonia and Ammonium sulphate, all remaining downstream processing steps can only remove water soluble impurities, and will consequently not remove any metal ions either adsorbed to or precipitated with the Aluminum hydroxide particles. No specific purification step to removal of contaminating metal ions is included in the manufacturing process.

The Ammonium sulphate solution is recycled following the precipitation and concentration of the Aluminum hydroxide gel.

1.3 Use of Aluminum hydroxide lots in JEV manufacturing

For the production of commercial JEV vaccine batches a total of 5 different Brenntag Alhydrogel® 2% lots have been used so far. These lots differed in the origin of the raw material Ammonia alum source and in the number of JEV batches they were used to formulate. An overview is given in Table 21.

2 Definitions & Abbreviations

Alhydrogel 2% Aluminum hydroxide solution (also referred to as alum)
DS/DP Drug Substance / Drug Product
ESG Environmental Scientifics Group
F-AAS Flame Atomic Absorption Spectrometry
FVL Final Vaccine Lot
GF-AAS Graphite Furnace Atomic Absorption Spectrometry
ICP-MS Inductively-Coupled-Plasma Mass Spectrometry
JEV Japan Encephalitis Virus
JE-PIV Japan Encephalitis Purified Inactivated Virus
LOQ Limit Of Quantification
P&TD Patch & Technical Development
PSD Particle Size Distribution
PZC Point of Zero Charge
QCI Quality Control Immunology

3 Materials and Methods

3.1 Alhydrogel® batches and raw materials

Alhydrogel® 2% lots: 3877, 4074, 4187, 4230, 4414, 4470, 4539, 4563, 4587, 4621 (not all Alum lots listed were used in the formulation of JE-PIV; see Table 21)

Alhydrogel® 2% 7x washed lots: 4577, 4580, 4596 (sourced from Brenntag, not typical of the 2% Alum received for formulation)

Ammonia Alum: AA0427, 6-80578-28, 10094, 10095, 91480 (represents ammonia alum used to produce different Alum lots. AA0427 was used to produce Alum lot #4230 used to formulate JEV09L37)

Ammonia 24% technical grade solution: 4140716; sample only and not related to any of the alum batches tested.

3.2 Alhydrogel® PSD Measurements

Aluminum hydroxide particle size distribution (PSD) was analyzed on a Malvern Mastersizer 2000μP system with a 20mL sample cell. Alhydrogel® 2% bulk substance was diluted 1:20 in water and 1mL was added to the sample cell. Final dilution of sample in sample cell was therefore 400 fold (0.005% Aluminum hydroxide).

3.3 Alhydrogel® Zeta-Potential Measurements

Zetapotential and point of zero charge (PZC) was measured on a Malvern Zetasizer ZS system equipped with a MPT-2 autotitrator. Alhydrogel® 2% bulk substance was diluted 1:20 in PBS and equilibrated over night at room temperature. For recording of the charge titration curve the pH was adjusted using 100mM HCl and 100mM NaOH solutions. PZC was determined by extrapolation of the zero charge in the titration plot (intersection of titration curve and x-axis). Point of zero charge corresponds to the pH value where the surface of the sample has no net charge.

3.4 Analysis of metal ions in Aluminum hydroxide and raw materials

Selected metal ions were analyzed either by inductively-coupled-plasma mass spectrometry (ICP-MS), flame atomic absorption spectrometry (F-AAS) and graphite furnace atomic absorption spectrometry (GF-AAS) at the Medical Laboratory Bremen (Germany). In short, samples containing Aluminum hydroxide were treated with conc. HNO3 under heat until a clear solution is obtained. The clear solution is then further diluted and analyzed. The presence and content of following metal ions were determined: Pb, Cd, Cr, Co, Fe, Cu, Ni, Ag, W and Al. Depending on the sample dilution, the limit of quantification (LOQ) was 5 to 25 ng/mL. Crystalline Ammonia Alum samples were dissolved in HNO3 before analysis. Ammonia had to be adjusted to acid pH before analysis.

In addition a semi-quantitative 70-Element scan was performed by ESG (UK) using a combination of ICP-MS (Agilent 7500ce) and ICP-AES (Perkin Elmer Optima 4300DV), which were calibrated using certified standards. The element scan is a screening method and not as sensitive as trace metal analysis for selected metals as performed by Medical Laboratory Bremen. However, such a screening gives a good overview about the presence and levels of certain metals.

4 Results

4.1 Determination of Alhydrogel® particle size distribution

Table 22 summarizes PSD data of two sublots each of Alhydrogel® lots 4230 and 4740. Distribution results are shown in Figure 11. Mean particle was ~2-4μm with populations of smaller (<1μm) and larger (>20μm) particles being present in all four samples. The four tested Alhydrogel® samples showed no significant difference in mean particle size distribution.

4.2 Zeta-Potential Measurements

Two sublots each of Alhydrogel® lots 4230 and 4740 (2% stock solution diluted 20 fold in PBS and equilibrated overnight at RT before analysis) were analyzed for point of zero charge. Table 23 summarizes results of PZC for the four samples showing very similar PZC in PBS buffer. Titration curves are shown in Figure 12. No difference in the titration curves and PZC could be observed between the four analyzed samples.

4.3 Determination of residual metal ion content in Alhydrogel® batches

The current limits for Fe in 2% Aluminum hydroxide solutions according to the Ph. Eur. are 15 ppm (= 15 µg/mL) and a total maximum of 20 ppm (= 20 µg/mL) for other heavy metals (such as Pb). However, a concentration of 15 ppm Fe would correspond to 0.27 mM Fe in solution. Taking into consideration that even trace amounts of residual metal ions can catalyze a variety of degradation reactions for proteins (e.g. oxidation, activation of proteases etc.) and that metals remain stable in solution, differences in metal ion content between Aluminum hydroxide lots might cause differences in antigen stability over time.

The concentrations of a number of metal ions in commercially available aluminum hydroxide lots were analyzed using ICP-MS. The results of these analyses are summarized in Table 24. Lots 4074, 4230, 4470, 4414 and 4539 were used in the production of commercial JEV batches. As a 2% Alhydrogel® stock solution equals an Al concentration of 10 mg/mL the quantification of Al content in the different samples can be used as reference for the results obtained for the other metal ions. Indeed an average Aluminum content of 10.3 mg/mL could be measured showing the accuracy and reproducibility of the method.

When comparing the different Alhydrogel® lots large variations in the amount of contaminating metal ions could be observed. Most notable contaminating

metal ions are Fe, Cr and Ni which were detected in all batches. In addition lot 4230 contained detectable amounts of Cu which was below LOQ in all other batches.

However, it has to be noted that none of these metals were detected in quantities near the specifications of Alhydrogel® mentioned above. For example the highest concentration of iron found in lot 4230 was 5.6 µg/mL or roughly 40% of the permitted concentration.

As the precipitation of Aluminum hydroxide during the process results in significant amounts of residual Ammonia in the final product, Brenntag Biosector offers a second grade of Alhydrogel®. In contrast to the standard product this "improved" Alhydrogel® is washed 7 times with water during the purification step instead of only 4 times for standard Alhydrogel®. To test if this additional washing steps would result in reduced metal ion contamination three different lots (4580, 4596 and 4577) were analyzed. Results are included in Table 24. No difference in metal ions comparing to the standard grade Alhydrogel® could be observed suggesting that the metal ions are either strongly bound to the surface of the Aluminum hydroxide particles or actually co-precipitate during the production process.

Figure 13 shows a comparison of the different Alhydrogel® lots analyzed. The total contaminating metal ion content for all tested contaminating elements are shown with the absolute shares for the three major metals Fe, Cr and Ni depicted in different colors. As can be seen lot 4074 has very little contaminating metal ions compared to the majority of the other analyzed batches. Only lot 3877 showed a similar lot contamination whereas lot 4230 shows by far the highest contamination of all batches analyzed during this investigation.

During the investigational testing a large variation in the metal ion content was observed between different batches of Alhydrogel® (see Figure 13). To test if these contaminating metal ions are located in the Aluminum hydroxide fraction or in the supernatant Lot 4230 was separated into a supernatant and a sediment fraction (see Table 25). As can be seen less than 2% of the metal ions could be detected in the supernatant indicating that all contaminating metal ions are either bound to the Aluminum hydroxide particle surface or inside the particle structures. The local metal ion concentration can therefore be estimated to be at least 50-100 times higher since the solid volume fraction (volume of Alum-pellet after centrifugation) of 0.1% Al2O3 (corresponding 0.5 mg/mL Al) used in JEV vaccine formulation is approx. 10-20µL per 1000µL of FVL.

4.4 Determination of residual metal ion content in Alhydrogel® raw materials

A total of 5 batches of Ammonia Alum (raw material for Alhydrogel®) were received from Brenntag Biosector. These batches were used for the production of 4 Alhydrogel® batches (see Table 26; note that 2 different batches of ammonia alum were used to produce Alum lot # 4563), three of them used for JEV vaccine lots. Upon delivery a marked difference in the visual appearance between the batches was observed. Whereas four samples showed a crystalline structure similar to large salt crystals one lot was more sand grain like. Furthermore whereas the crystals were completely colorless the Ammonia Alum AA0427 material was zinc-white. This finding is of importance as lot AA0427 was used for the production of Alhydrogel® batch 4230 which was subsequently used in the production of JEV09L37 (see Table 26). However, according to Ph. Eur. the appearance of Alum can be “granular powder or colourless, transparent, crystalline masses” (European Pharmacopoeia 7th edition. Monograph 1357p). Consequently, all five raw materials comply with this definition.

ICP-MS analysis of these five Ammonia Alum lots and one lot of 24% Ammonia solution (technical grade) showed that the lot 6-80578-28 has very low amounts of contaminating metal ions whereas lot AA0427 has very high amounts (see Table 27). This correlates with the analysis of the corresponding Alhydrogel® batches (see Table 24) showing that the origin of these metal ions is in the raw material and not process derived.

Figure 14 shows a comparison of the different Ammonia Alum lots analyzed. The total metal ion content for all tested contaminating elements are shown with the absolute shares for the three major metals Fe, Cr and Ni depicted in different colors. Ammonia Alum lot 6-80578-28 has ~ 40 times less contaminating metal ions compared to lot AA0427 at identical Al concentration resulting in the observed much higher purity of the corresponding final Alhydrogel® batches 4074 and 4230.

When comparing the results for the three Ammonia Alum lots with their corresponding Alhydrogel® lots a similar ratio for the three major contaminations Fe, Ni and Cr can be observed (Table 28).

This shows that there is basically no change in the metal ion composition during the whole Aluminum hydroxide production process. Consequently, a secondary contamination during the production process (e.g. other chemicals or the storage container) can be excluded.

Table 29 summarizes results of semi-quantitative element scan analysis performed by ESG.

5 Summary

Alhydrogel® is used in a 0.1% final concentration as adjuvant in the current JEV vaccine formulation. During the investigation of an out-of-specification (OOS) potency result for production FVL JEV09L37 an evaluation of the Alhydrogel® production process was initiated. A total of 13 different Alhydrogel® lots were analyzed for the presence of contaminating metal ions that could reduce protein stability.

Large variations in the concentration of a number of metal ions were observed for different Alhydrogel® lots. When analyzing the raw materials it was shown that these contaminations were present at the same concentration as found within the Alhydrogel.

Higher levels of Fe, Ni and Cu ions were noted in Alhydrogel® lot 4230 when compared to the other investigated lots. Lot 4230 was the only one where residual Cu ions were detected. This lot 4230 was used for the formulation of FVL JEV09L37.

When analyzing supernatant and insoluble fraction of an Alhydrogel® batch these contaminating metal ions could only be found in the precipitate indicating that these ions are either bound to the Aluminum hydroxide particle surface or actually part of the particle.

The raw material used for batch 4230 showed an optical difference to the other batches used in JEV vaccine production. Although macroscopic and in composition different from other Alhydrogel® lots used for JEV production, lot 4230 fulfilled all requirements detailed by the Ph. Eur. Also physical characterization (particle size distribution and point of zero charge) showed no differences between lot 4230 and other Alhydrogel® lots not showing these high metal ion contaminations.

Example 4

1.1. Materials, Equipment and Methods

1.2. Equipment

Analytical balance (readability of 0.1mg; e.g. Mettler Toledo XP205DR/M)

Precision balance (readability of 0.1g; e.g. Mettler Toledo, Model N° XS6002S Delta Range)

Filter Units 0.22µm (e.g. Stericup Cat N° SCGPV01RE) or 0.2µm filter system (e.g. 50mL Millipore Steriflip)

Freezer (- 20°C) and Ultra-Freezer (-80°C)

Fridge (+ 2 to 8°C)

Magnetic stirrer (e.g.KIKA Labortechnik RCT basic) and magnetic stir bars

Microplate Washer: e.g. BioTek ELx405

Microplate Reader: e.g. BioTek Synergy 2 and Gen5 Secure software

Microplate Incubator (37°C)

Microtiter Sealing tape (e.g. Thermo Electron 9503130)

Multichannel pipettes and tips (e.g. Eppendorf Research Pro 50-1200µL, Eppendorf Research, 10-100µL)

pH meter (e.g. WTW Series ino Lab, Terminal 740 and pH/Cond. 740)

Pipettes and tips (e.g. Eppendorf Research, 0.5-10µL, 2-20µL, 20-200µL, 100-1000µL, 500-5000µL)

Pipettor (e.g. IBS Biosciences Pipetboy)

PP Tubes 15mL (e.g. Sarstedt 62.515.006) or PP tubes 50mL (e.g. Greiner 227261)

Reagent Reservoir 50mL (e.g. Corning Incorporated 4870)

Serological pipettes (e.g. Falcon, 2mL, 5mL, 10mL, 25mL, 50mL)

Titertube Micro Tubes – Bulk (BioRad 223-9391)

Vortex mixer (e.g. VWR Analog Vortex Mixer, Model N°945304)

1.5mL or 2.0mL Eppendorf LoBind tubes (Cat N° 0030 108.116, CatN° 0030 108.132, respectively)

96 well Microplate (F96 Cert. Maxisorp Nunc-Immunoplates)

For analysis of DP samples in addition:

Bench top centrifuge (e.g. Beckman coulter, Microfuge 16 Centrifuge, Cat N°A46473)

Orbital shaker (e.g. Eppendorfer Thermomixer compact)

50mL PP tubes (e.g.Greiner 227261)

1.3. Reagents

PBS 10x (e.g. Gibco, Cat.N° 14200-083)

Tween 20 (e.g. Sigma Cat. N° P7949)

2M Sulphuric Acid (Volumetric solution, e.g. Fisher, Cat. No. J/8410/17)

De-ionised water, e.g. (Milli-Q, 18.2Ω)

Sodium carbonate – bicarbonate capsules (e.g. Sigma, Cat. No. C3041)

Hydrochloric acid (HCl) 1mol/L (e.g. Merck, Cat.N° 1.09057.1000)

Sodium hydroxide (NaOH) 1mol/L (e.g. Merck, Cat. N°1.09132.1000)

Glycerol (e.g. Sigma)

For analysis of DP samples in addition:

Di-potassium hydrogen phosphate trihydrate (e.g. Sigma, Cat No. P5504)

Potassium di-hydrogen phosphate (e.g. VWR, AnalaR Normapur, Cat No. 26936.260)

Albumin, Bovine Serum (BSA), ELISA grade (e.g. Sigma, Cat. No. A3059)

TMB Substrate (e.g. BioFX, TMBW-1000-01)

Donkey anti rabbit IgG HRP Conjugate (Jackson Immuno Research, Cat N° 711-035-152)

Reconstitution:

The content of 1 vial (0.4mg) is reconstituted in 0.5mL of de-ionised water and thoroughly mixed until total dissolution. Add 0.5mL of Glycerol and mix it further until homogeneity. Aliquots are stored at -20°C until use.

Inactivated JEV Reference Standard (Intercell Biomedical Ltd.)

Purified sheep anti-JEV (Intercell Biomedical Ltd.)

Purified rabbit anti-JEV (Intercell Biomedical Ltd.)

1.4. Solutions

a) 0.05M carbonate buffer at pH 9.6 (used for coating of ELISA plates)

For 100mL buffer, dissolve one bi-carbonate /carbonate buffer capsule in 100mL de-ionised water. Check the pH and adjust to 9.6 ± 0.1 with HCl or NaOH if required. Use on the day of preparation only. Keep ELISA coating buffer at RT during the day of use, then discard.

b) ELISA wash buffer and part of block / sample diluent (PBS-T)

Prepare approximately 1 litre for every plate used. Dilute 10x PBS stock 1+9 in de-ionised water, mix well and check pH (7.4 +/- 0.1), adjust with 1M HCl or 1M NaOH as required. Add 0.05% (v/v) Tween20, mix well.

e.g. ELISA wash buffer (PBS-T) [1L]:

100mL 10x PBS

900mL de-ionised water

Mix well, check/adjust pH (7.4 +/- 0.1).

0.5mLTween20

Mix well.

Use on the day of preparation only; keep ELISA wash buffer at RT during the day of use, then discard.

c) Blocking solution: 5% BSA in PBS-T

Prepare approximately 25mL for every plate. Measure required quantity of PBS-T into a clean glass bottle using a serological pipette. Add a clean magnetic stir bar. Weigh the required amount of BSA, add to the surface of the PBS-T and mix gently on a magnetic stirrer until all the BSA has gone into solution. Filter solution using a 0.2µm filter (either Steriflip filter system or syringe filter).

e.g. Blocking solution [100mL]

5g BSA

100mL PBS-T

Use on the day of preparation only; keep blocking solution at RT during the day of use then discard.

d) Sample diluent: 1% BSA in PBS-T

Prepare as above but using 1g of BSA per 100mL PBS-T, approximately 25mL is required per plate.

e.g. Sample diluent [100mL]

1g BSA

100mL PBS-T

Use on the day of preparation only; keep sample diluent at RT during the day of use, then discard.

<u>For analysis of DP samples in addition:</u>

e) 1x PBS

Prepare 1 part 10x PBS with 9 parts de-ionised water

e.g. 1x PBS [100mL]

10mL 10x PBS

90mL de-ionised water

Use on the day of preparation only; keep 1x PBS at RT during the day of use, then discard.

f) 20x ELISA buffer

Weigh an appropriate amount of BSA into a suitable container to make a 20x solution. Add the appropriate volume of 1x PBS. Add Tween20 to a final concentration of 0.05% Mix on the magnetic stirrer until the BSA is fully dissolved. Filter the solution through a 0.2µm filter (using either Steriflip filter system or syringe filter) into a sterile container (and aliquote as needed).

e.g. 20x ELISA Buffer [25mL]

5g BSA

25mL 1xPBS

12.5µL Tween20

The solution can be stored at +2-8°C for 1 week.

g) 2x ELISA buffer

It is prepared by dilution of the 20x ELISA Buffer with 1x PBS (1 part 20x ELISA Buffer and 9 part 1x PBS).

e.g. 2x ELISA Buffer [20mL]

2mL 20x ELISA Buffer

18mL 1x PBS

Use on the day of preparation only; keep 2x ELISA buffer at RT during the day of use, then discard.

h) Desorption buffer

o Potassium phosphate stock solution: Make a 3x stock solution of potassium phosphate (2.4M) by dissolving the appropriate volume of di-potassium phosphate trihydrate and of potassium dihydrogen phosphate in de-ionised water. Place on a magnetic stirrer and once dissolved make up the required volume, check that the pH of the solution is 8.0+/- 0.1. Filter through a 0.2µm filter.

e.g. 3x stock solution of Potassium phosphate (2.4M) [50mL]

23.963g	Di-potassium phosphate trihydrate
2.041g	Potassium dihydrogen phosphate
Make up to 50mL	De-ionised water

Store at +2°-8°C for up to 1 month.

o Make working strength desorption buffer (0.8M potassium phosphate buffer containing 1% BSA and 0.05% Tween20) by adding the appropriate volume of potassium phosphate stock (2.4M), of Tween20 and of BSA to the required volume of de-ionised water. Mix thoroughly and use on the day of preparation.

e.g. working strength desorption buffer [15mL]

5mL	Potassium Phosphate (2.4M)
7.5µL	Tween20

0.15g	BSA
10mL	De-ionised water

Keep working strength desorption buffer at RT during the day of use.

1.5. Test samples and antibodies

Test samples:

- Drug Substance and / or NIV (various batches)
- JEV Vaccine samples (final bulk vaccine and final vaccine lot)

Inactivated JEV Reference Standard (Neutralised Inactivated Virus – NIV) (Intercell Biomedical Ltd.)

Polyclonal Antibodies:

- Coating antibody: Purified Sheep anti-JEV (Intercell Biomedical Ltd.)
- Primary detection antibody: Purified Rabbit anti-JEV (Intercell Biomedical Ltd.)

Secondary conjugated antibody: Donkey anti-Rabbit HRP Conjugate (Jackson Immuno Research Cat. N° 711-035-152)

2 Procedure

2.1. Plate coating

- Label the plate with plate number, date and analyst.
- Prepare fresh 0.05M carbonate buffer (pH 9.6) on the day of plate coating. Allow approximately 12mL for each plate coated.
- Remove the required number of aliquots of the coating antibody from the freezer and allow thawing at RT. Prepare a dilution of Purified Sheep anti-JEV in carbonate buffer. Mix well by inversion of the tube.
- Using the multichannel pipette, apply 100µL/well to a 96-well Maxisorp plate within 15min of preparation of the antibody dilution.
- Cover with microtiter sealing tape and incubate 17 to 72hrs at + 2-8°C.

2.2. Washing

o Remove plate from the refrigerator and allow warming to room temperature.

o Wash the plate/s with the Microtiter plate washer 3 times using the respective wash program (300µL per well, three times, final dispense). After that: remove any remaining wash buffer by decanting. Invert the plate and blot it against a clean paper towel. Do not allow microtiter plate to dry between wash steps and reagent addition.

2.3. Blocking

o Prepare a Blocking Solution 5% (w/v) of BSA in PBS-T as above.

o Apply 200µL Blocking Solution per well, cover the plate(s) with a cover plate and incubate at 37°C for 1 hour +/- 10 min.

2.4. Preparation of Standard Curve Dilutions

o Remove the NIV reference standard from the freezer, allow thawing at RT, mix well. Prepare a 1AU/mL stock dilution of the current reference standard; use at least 20µL of NIV reference standard for dilution.

e.g. NIV reference standard Pre-dilution:

Concentration: 235AU/mL (lot N° 03/2009)

To prepare a 1AU/mL working standard solution dilute it 1 to 235 in sample diluent:

4680 µL sample diluent

20 µL NIV reference standard

o Prepare then the following working standard solutions from the 1AU/mL pre-dilution:

0.8 AU/mL, 0.6 AU/mL, 0.4 AU/mL, 0.2 AU/mL, 0.1 AU/mL and 0.05 AU/mL in sample diluent.

2.5. Quality Control Samples

a) Quality control (QC) samples (for example at 0.75, 0.30 and 0.18 AU/mL) should be made from the NIV reference standard pre-dilution freshly at the time of the assay then discarded once used.

b) These controls are part of the system suitability criteria and allow the performance of the assay to be monitored over time.

2.6. Preparation of Test Samples

Drug Substance Preparation

Drug substance test samples are received for testing at unknown concentrations. These will be tested at six dilutions in triplicate. The dilutions will be made independently into the range of the standard curve, e.g. pre-dilution of 1 in 15 or other suitable dilution then six dilutions with sample buffer.

NIV Sample Preparation

NIV samples will be received for testing at unknown concentrations and pre-diluted in the range of the standard curve (e.g. 1 in 30 or other suitable dilution) then diluted six times in the same manner as the DS samples.

Drug Product Supernatant Preparation

a) For the analysis of Bulk-DP samples mix well sample by vortexing. Transfer exactly 1mL into a 1.5mL LoBind Eppendorf tube.
For the analysis of final product container samples transfer the content of 2 syringes (0.6mL per syringe) of the same lot into a 1.5mL LoBind Eppendorf Tube. Mix content of tube thoroughly by inversion to ensure homogeneity of the DP and transfer exactly 1mL into fresh 1.5mL LoBind Eppendorf tube.

b) Centrifuge tubes containing 1mL DP each at 3300xg for 5 minutes.

c) For each sample, pipette 25µL of 20x ELISA buffer into fresh LoBind Eppendorf tube.

d) Carefully remove 475µL of the supernatant without disturbing the alum pellet and transfer into the tube containing the 20x ELISA buffer. Mix gently by inversion. Re-spin 2 min at 16,000xg. Store sample at +2-8°C prior to analysis.

NOTE: DP supernatant samples prepared in this way should be measured neat in triplicate in the inactivated JEV ELISA.

e) Carefully remove as much of the residual supernatant from the centrifuged tube using a 10-200µL pipette without disturbing the alum pellet and discard the supernatant.

f) The pellet obtained is subjected to the Desorption procedure as described below.

Drug Product Desorption Procedure

a) Add 158µL of working strength desorption buffer to each pellet left in the LoBind tube.

b) Resuspend the pellet by pipetting up and down several times to ensure complete re-suspension of the pellet and homogenisation of the sample.

c) Incubate samples for 10 min at RT on an orbital shaker at 500rpm.

d) After incubation centrifuge samples at 3300xg for 5minutes.

e) For each sample pipette 250µL of 2x ELISA buffer into a fresh LoBind Eppendorf tube.

f) Carefully remove 83.3µL from the upper part of the supernatant containing the desorbed product without disturbing the pellet and transfer into the tube containing the 2x ELISA buffer. Remove remaining supernatant using a 20-200µL pipette without disturbing the pellet and discard the supernatant.

g) Add another 158µL of working strength desorption buffer to each pellet.

h) Carry out 2 more desorption cycles (3 in total) pooling the 3x 83.3µL of the desorbed material + 250µL ELISA buffer into the appropriate tube. After the last step the remaining pellet can be discarded.

i) The final concentration of the viral antigen in the desorbed pool(s) should now be the same as the original 1mL of DP from which it was desorbed. Therefore, the concentration of inactivated JEV antigen content measured in the desorbed pool can be directly related to the original DP.

Note: Analyse the desorbed samples by ELISA on the day of desorption.

j) Dilution of desorbed DP samples:

These will be tested at six dilutions in triplicate. An appropriate pre-dilution will be performed in the range of the standard curve e.g. 1 in 15 (100µL to 1400µL diluent) or other suitable dilution, then six dilutions of 1 in 15 pre-dilution will be made independently using sample diluent.

2.7. Sample Loading and Plate Plan

Prepare samples and standards before analysis.

After blocking wash the plate using the plate washer employing the JEV ELISA program. After that, remove any remaining wash buffer by decanting. Invert the plate and blot it against a clean paper towel. Do not allow microtiter plate to dry between wash steps and reagent addition.

Add 100µL/well of standards / controls / samples and cover with cover plate and incubate for 1 hour+/- 10 min at 37°C.

Add 100µL of sample diluent to all wells not required for testing.

2.8. Preparation of Primary Antibody

Remove the required number of aliquots of the primary antibody from the freezer and allow to thaw at RT. Prepare max 15 min before use Rabbit anti-JEV in sample diluent at a suitable dilution. Following sample incubation, wash the plate using the plate washer employing the JEV ELISA program. After that, remove any remaining wash buffer by decanting. Invert the plate and blot it against a clean paper towel. Do not allow microtiter plate to dry between wash steps and reagent addition.

Add 100µL/well of diluted primary antibody, cover with cover plate and incubate for 1 hour +/- 10 min at 37°C.

2.9. Preparation of Secondary Antibody Conjugate

Remove the required number of aliquots of the secondary antibody conjugate from the freezer and allow to thaw at RT. Prepare max 15 min before use a dilution of Donkey anti-Rabbit-HRP in sample diluent; e.g. for a 1 in 10,000 dilution for make a 1 in 100 pre-dilution then make a second dilution of 1 in 100.

Following primary antibody incubation, wash the plate using the plate washer employing the JEV ELISA program. After that, remove any remaining wash buffer by decanting. Invert the plate and blot it against a clean paper towel. Do not allow microtiter plate to dry between wash steps and reagent addition. Add 100µL/well of diluted secondary antibody conjugate, cover with cover plate and incubate for 1 hour +/- 10 min at 37°C.

2.10. Substrate Incubation

When the conjugate has been added, remove TMB from the 2-8°C refrigerator. Pipette the required volume (12mL of TMB per plate) into a 50mL centrifuge tube, using a serological pipette. Allow the TMB to reach room temperature in the dark. Following conjugate incubation wash the plate 3 times with the plate washer employing the JEV ELISA program. After that: remove any remaining wash buffer by decanting. Invert the plate and blot it against a clean paper towel. Do not allow microtiter plate to dry between wash steps and reagent addition. Add 100µL/well of TMB and develop the plate in the dark at Room Temperature for 10 minutes.

2.11. Stopping and Reading

After 10 minutes of TMB incubation, stop the development by adding 100µL/well 2M sulphuric acid. Read the plate at 450nm (reference filter

630nm) within 10 minutes of stopping using the BioTek reader and Gen5 Secure software.

2.12. Data analysis

NIV/DS data analysis:

Gen5Secure software will be used to calculate the % Recovery of the QCs, concentration x dilutions, mean concentration of the samples corrected for dilutions and this value multiplied by 1.05 to correct for the addition of ELISA buffer.

DP data analysis:

Gen5Secure software will be used to calculate the % Recovery of the QCs, concentration x dilutions and mean concentration of the dilutions for the samples.

For DP Supernatant samples:

If the concentration of the supernatant sample is below the LLOQ of the assay (i.e. 0.05AU/ml), then the supernatant sample should be recorded as <0.05 AU/ml

If the concentration of the supernatant sample is within the LOQs of the assay (i.e. 0.05AU/ml to 1.25AU/ml), then the concentration value is recorded for the supernatant sample.

If the concentration of the supernatant sample is above the ULOQ of the assay (i.e. 1.25AU/ml), then the preparation of the drug product supernatant should be repeated. The supernatant sample will be re-tested by performing a suitable pre-dilution into the range of the standard curve followed by 6 sample dilutions. (The desorbed Drug Product sample does not need to be repeated.) The mean concentration for the dilutions that are within the LOQs of the

assay (LLOQ 0.05AU/ml to 1.25AU/ml) will be the recorded concentration value for the supernatant sample, provided that the system suitability are met and at least 4 out of the 6 sample dilutions are within the LOQs.

2.13. Assay Acceptance Criteria

a) The correlation coefficient for the calibration curve must be > 0.980.

b) %CVs ≤15% for standards and samples (except DP supernatant) for the four highest concentrations of the dilutions, %CV ≤15% for controls

c) Individual blank ODs must be ≤ 0.2.

d) Assay controls must be within specified defined limits (for freshly prepared controls 2 out of 3 QCs should have observed concentrations within ± 30% of the nominal values; OR the levels set during QC qualification) for the assay to pass.

e) Assay validity will be recorded on the Gen5-print-out. If the plate fails to meet the defined acceptance criteria the assay is deemed invalid.

2.14. Reporting of data

NIV

a) Antigen content

The reported value for inactivated AU/mL is the mean of the concentrations (which are within the LOQs of 0.04 to 1.25AU/mL) calculated for the single sample dilutions corrected by the respective dilution factors, and the mean multiplied by a correction factor of 1.05 to account for the 5% volume of 20x ELISA buffer that was added to each sample when it was taken. Antigen content will be recorded on Gen5 print-out.

DS:

a) Identity

If the absorbances of the samples at lowest dilution (highest concentration) are higher than 3 standard deviations above the mean value of the blank the result will be reported as positive

b) Antigen content

The reported value for inactivated AU/mL is the mean of the concentrations (which are within the LOQs of 0.04 to 1.25AU/mL) calculated for the single sample dilutions corrected by the respective dilution factors, and the mean multiplied by a correction factor of 1.05 to account for the 5% volume of 20x ELISA buffer that was added to each sample when it was taken. Antigen content will be recorded on Gen5 print-out.

Desorbed DP:

a) Identity:

If the absorbances of the samples at lowest dilution (highest concentration) are higher than 3 standard deviations above the mean value of the blank the result will be reported as positive.

b) Antigen Content

The reported value for inactivated AU/mL is the mean of the concentrations (which are within the LOQs of 0.05 to 0.8AU/mL) calculated for the single sample dilutions corrected by the respective dilution factors. Antigen content will be recorded on Gen5 print-out.

DP supernatant (degree of adsorption / degree of non-adsorption):

Degree of adsorption is reported in relationship to aluminium hydroxide formulated drug substance post filtration.

a) For calculation of the reported value, the reported antigen content (AU/mL) for the respective DS sample (post filtration) corrected for the dilution with aluminium hydroxide (5%) will be set at 100% and the percentage of the concentration measured in the supernatant (corrected for the addition of 5% ELISA buffer) calculated in relation to that. The reportable value will be the difference between 100% and the percentage calculated for the supernatant. Results will be reported to 2 decimal places.

$$\text{Degree of adsorption (\%)} = 100\% - \frac{\text{DP supernatant (AU/mL)} * 1.05}{\text{DS (AU/mL)} * 0.95} * 100\%$$

The degree of non-adsorption will be calculated as detailed below and results will be reported to 2 decimal places:

$$\text{Degree of non-adsorption (\%)} = \frac{\text{DP supernatant (AU/mL)} * 1.05}{\text{DS (AU/mL)} * 0.95} * 100\%$$

b) In case the neat supernatant does not contain any measurable antigen (ie. observed supernatant concentration less than LLOQ, where LLOQ = 0.05 AU/mL), the LLOQ will be used for the calculation of the result. The result in this case is reported as "greater than x%". For example if the DS sample is measured as 12.00AU/mL and no signal was measured in the supernatant; with the LLOQ of 0.05AU/mL then amount in supernatant is <0.05*1.05 = < 0.0525 AU/mL. The amount of DS after buffer correction is 12.00*0.95 = 11.40AU/mL, and the reported result for degree of adsorption is < 100-0.0525/11.4*100 = > 99.54%. The degree of non-adsorption will also be reported (i.e. 100 - the % degree of adsorption).

Example 5

Introduction:

In order to further investigate the mode of action that leads to product instability/potency loss of the JEV vaccine, Ala-(His)6-OprF190-342-OprI21-83 (SEQ ID NO: 1, Figure 15) – herein also referred to as "protein A" was used in

a preeliminary screening assay incorporating alhydrogel lots with different metal content and spiking with copper ions and sulfite.

Material:

- Copper(II)chloride dihydrate (Sigma, Order no. 807483)
- 10xPBS (Gibco, Order No. 14200-091)
- 15ml Falcon tubes(Greiner, Cat. No. 188724)
- Incubator Infors HT Incubator Multitron Standard (InforsAG)
- Aqua bidest. (Fresenius Kabi, Art no. 0712221/01 A)

Preparation of stock solutions:

- Copper(II) stock solution

  20 mM Copper(II) stock solution was prepared by dissolving 341 mg of Copper(II)chloride dihydrate in 100 mL of aqua bidest..

- Sodiummetabisulfite stock solution

  200 mM Sodiummetabisulfite stock solution was prepared by dissolving 1.52 g of Sodiummetabisulfite in 35 mL PBS. This solution was adjusted the pH to 7.3 with NaOH and filled up to a volume of 40 mL with PBS. The solution was them filtered via 0.2µ syringe filter.

Preparation of working solutions

Working solutions were prepared by dilution of metal stock solutions with aqua bidest. and sterile filtration via 0.2µ syringe filter. (Mini Kleenpak 25mm- Pall)

Preparation of buffer solutions

- ⅓ PBS + 0.9% NaCl

  1 x PBS buffer solution was prepared by 1:10 dilution of 10 x PBS with aqua bidest.. The pH of this buffer solution was 7.5. PBS buffer

solutions adjusted to pH 7.3 and 8.0 were prepared by adjusting the pH with HCl or NaOH respectively.

9 g of NaCl were dissolved in 333mL of either pH 7.3 of pH 8.0 buffer solution and then brought to 1000 mL with aqua bidest.. followed by filtration via 0.2µ bottletop filter.

Sample preparation:

Formulations of Protein A and different lots of alhydrogel (Lot 4230 & Lot 4074) were prepared in ⅓ PBS + 0.9% NaCl at two different pH values and were spiked with sulfite according to the following scheme:

Sample				Spike	
No.	Name	pH	Alum batch	Cu(II) [ng/mL]	Sulfit [mM]
1	17112011_PROTEIN A_4074_ref_4°_pH7.3	7.3	4074		
2	17112011_PROTEIN A_4074_ref_37°_pH7.3	7.3	4074		
3	17112011_PROTEIN A_4074_Sulfit_37°_pH7.3	7.3	4074		1
4	17112011_PROTEIN A_4230_ref_4°_pH7.3	7.3	4230		
5	17112011_PROTEIN A_4230_ref_37°_pH7.3	7.3	4230		
6	17112011_PROTEIN A_4230_Sulfit_37°_pH7.3	7.3	4230		1
7	17112011_PROTEIN A_4074_ref_4°_pH8	8	4074		
8	17112011_PROTEIN A_4074_ref_37°_pH8	8	4074		
9	17112011_PROTEIN A_4074_Sulfit_37°_pH8	8	4074		1
10	17112011_PROTEIN A_4230_ref_4°_pH8	8	4230		
11	17112011_PROTEIN A_4230_ref_37°_pH8	8	4230		
12	17112011_PROTEIN A_4230_Sulfit_37°_pH8	8	4230		1

Samples 1, 6, 11 and 16 were stored at 4°C (reference samples). All other samples were incubated at 37°C for 96 hours.

After 96 hours all samples were subjected to a desorption procedure to separate the antigen from alhydrogel. The desorbed antigen was analyzed by RPC.

Results:

Results showed severe degradation of the antigen Protein A in the presence of sulfite. The degradation was more pronounced in the samples formulated with alhydrogel of higher metal impurity content.

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Table 1. Metal ion content in Aluminium hydroxide lot 4230 and 4074 analyzed by ICP-MS.

Alum Lot (2% solution)	**Al**	**Cr**	**Fe**	**Co**	**Ni**	**Cu**	**Ag**	**Cd**	**W**	**Pb**	**V**	**Rb**	**Mo**
	µg/mL	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**	**ng/mL**
Alum Lot 4230 RQCS 0890	9570	1139	5640	7	816	64	<5	<5	<25	24	13	<5	11
Alum Lot 4074 RQCS0013	9130	20	266	<5	15	<25	<5	<5	<25	19	<5	<5	<5
Mix 50/50% of both Lots*	9130	579	2952	<5.8	415	<45	<5	<5	<25	21.6	<9	<5	<8

*Calculated residual metal content

Table 2: Pipetting scheme of DOE

Sample				%	%	µg/mL	U	ppm
No.	Name	pH	NIV dil	Alum 4230	Alum 4074	PS Frag. Stock	Extractables Stock	CH2O
1	20110819_DOE_spl_1	7	2	100	0	50	4.2	0
2	20110819_DOE_spl_2	7	2	0	100	0	4.2	0
3	20110819_DOE_spl_3	7	2	0	100	0	0	40
4	20110819_DOE_spl_4	8	2	0	100	50	4.2	0
5	20110819_DOE_spl_5	7.5	2	50	50	50	0	0
6	20110819_DOE_spl_6	7	2	0	100	50	0	40
7	20110819_DOE_spl_7	7	2	100	0	50	0	0
8	20110819_DOE_spl_8	8	2	0	100	50	0	0
9	20110819_DOE_spl_9	8	2	100	0	0	0	0
10	20110819_DOE_spl_10	8	2	100	0	50	0	0
11	20110819_DOE_spl_11	8	2	0	100	50	4.2	40
12	20110819_DOE_spl_12	7	2	100	0	0	4.2	0
13	20110819_DOE_spl_13	8	2	0	100	0	0	40
14	20110819_DOE_spl_14	7	2	100	0	0	0	40
15	20110819_DOE_spl_15	8	2	0	100	0	4.2	0

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Table 2 continued

16	20110819_DOE_spl_16	8	2	100	0	50	0	40
17	20110819_DOE_spl_17	7	2	0	100	50	4.2	0
18	20110819_DOE_spl_18	7	2	0	100	50	4.2	40
19	20110819_DOE_spl_19	8	2	100	0	0	4.2	40
20	20110819_DOE_spl_20	8	2	0	100	0	4.2	40
21	20110819_DOE_spl_21	8	2	0	100	50	0	40
22	20110819_DOE_spl_22	8	2	100	0	0	4.2	0
23	20110819_DOE_spl_23	7	2	0	100	0	4.2	40
24	20110819_DOE_spl_24	7	2	100	0	50	0	40
25	20110819_DOE_spl_25	7	2	0	100	50	0	0
26	20110819_DOE_spl_26	7	2	0	100	0	0	0
27	20110819_DOE_spl_27	8	2	100	0	50	4.2	0
28	20110819_DOE_spl_28	8	2	100	0	0	0	40
29	20110819_DOE_spl_29	8	2	100	0	50	4.2	40
30	20110819_DOE_spl_30	7	2	100	0	0	0	0
31	20110819_DOE_spl_31	7.5	2	50	50	0	0	0
32	20110819_DOE_spl_32	8	2	0	100	0	0	0
33	20110819_DOE_spl_33	7	2	100	0	50	4.2	40
34	20110819_DOE_spl_34	7	2	100	0	0	4.2	40

Table 2 continued

Sample			Volume [µl]							
No.	Name	pH	NIV	Buffer	Alum 4230	Alum 4074	PS Frag. Stock	Extractables Stock	CH2O	Total
1	20110819_DOE_spl_1	7	2500	1812	250	0	125	313	0	5000
2	20110819_DOE_spl_2	7	2500	1937	0	250	0	313	0	5000
3	20110819_DOE_spl_3	7	2500	2228	0	250	0	0	22	5000
4	20110819_DOE_spl_4	8	2500	1812	0	250	125	313	0	5000
5	20110819_DOE_spl_5	7.5	2500	2125	125	125	125	0	0	5000
6	20110819_DOE_spl_6	7	2500	2103	0	250	125	0	22	5000
7	20110819_DOE_spl_7	7	2500	2125	250	0	125	0	0	5000
8	20110819_DOE_spl_8	8	2500	2125	0	250	125	0	0	5000
9	20110819_DOE_spl_9	8	2500	2250	250	0	0	0	0	5000
10	20110819_DOE_spl_10	8	2500	2125	250	0	125	0	0	5000
11	20110819_DOE_spl_11	8	2500	1790	0	250	125	313	22	5000
12	20110819_DOE_spl_12	7	2500	1937	250	0	0	313	0	5000
13	20110819_DOE_spl_13	8	2500	2228	0	250	0	0	22	5000
14	20110819_DOE_spl_14	7	2500	2228	250	0	0	0	22	5000
15	20110819_DOE_spl_15	8	2500	1937	0	250	0	313	0	5000
16	20110819_DOE_spl_16	8	2500	2103	250	0	125	0	22	5000

Table 2 continued

17	20110819_DOE_spl_17	7	2500	1812	0	250	125	313	0	5000
18	20110819_DOE_spl_18	7	2500	1790	0	250	125	313	22	5000
19	20110819_DOE_spl_19	8	2500	1915	250	0	0	313	22	5000
20	20110819_DOE_spl_20	8	2500	1915	0	250	0	313	22	5000
21	20110819_DOE_spl_21	8	2500	2103	0	250	125	0	22	5000
22	20110819_DOE_spl_22	8	2500	1937	250	0	0	313	0	5000
23	20110819_DOE_spl_23	7	2500	1915	0	250	0	313	22	5000
24	20110819_DOE_spl_24	7	2500	2103	250	0	125	0	22	5000
25	20110819_DOE_spl_25	7	2500	2125	0	250	125	0	0	5000
26	20110819_DOE_spl_26	7	2500	2250	0	250	0	0	0	5000
27	20110819_DOE_spl_27	8	2500	1812	250	0	125	313	0	5000
28	20110819_DOE_spl_28	8	2500	2228	250	0	0	0	22	5000
29	20110819_DOE_spl_29	8	2500	1790	250	0	125	313	22	5000
30	20110819_DOE_spl_30	7	2500	2250	250	0	0	0	0	5000
31	20110819_DOE_spl_31	7.5	2500	2250	125	125	0	0	0	5000
32	20110819_DOE_spl_32	8	2500	2250	0	250	0	0	0	5000
33	20110819_DOE_spl_33	7	2500	1790	250	0	125	313	22	5000
34	20110819_DOE_spl_34	7	2500	1915	250	0	0	313	22	5000
		sum:	85000	69014	4250	4250	2125	5015	346	170000

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Table 3: Comparison of leachables from stopper extract and JEV09L37 SN. All peaks with an area of > 0.1mAU.min are included in this table.

Retention time	**Stopper extract concentrate**	**Stopper extract concentrate 1:16 diluted in formulation**	**FVL L37 SN**	**Relative concentration compared to FVL**
(min)	**Peak area (mAU.min)**			**(%)**
12.40	0.79	0.05	0.10	47
13.14	1.91	0.12	n.d.	additional peak compared to FVL
13.48	0.81	0.05	n.d.	additional peak compared to FVL
13.74	0.43	0.03	n.d.	additional peak compared to FVL
14.10	0.75	0.05	n.d.	additional peak compared to FVL
14.41	0.49	0.03	0.25	12
16.12	29.64	1.85	0.45	414
16.71	2.75	0.17	n.d.	additional peak compared to FVL
17.15	14.68	0.92	0.11	815
18.68	0.90	0.06	0.57	10
20.08	0.70	0.04	0.25	18
20.75	0.42	0.03	n.d.	additional peak compared to FVL
21.27	0.54	0.03	n.d.	additional peak compared to FVL
22.13	2.84	0.18	0.15	122
22.74	0.55	0.03	0.17	20
23.74	1.74	0.11	0.27	41
24.88	0.98	0.06	0.12	51
27.63	0.84	0.05	0.16	32
29.46	0.72	0.04	n.d.	additional peak compared to FVL
31.40	0.39	0.02	n.d.	additional peak compared to FVL
35.23	4.70	0.29	0.41	72
SUM	**67.59**	**4.2**	**3.0**	**140**

Table 4: DOE results obtained after 4 and 8 weeks at 22°C. Antigen was desorbed from Alum and analysed by ELISA (monoclonal and polyclonal). * (x-fold increase compared to FVL)

						4 weeks at 22°C			8 weeks at 22°C		
Sample	pH	Alum 4230 (%)	Spiked PS Frag. (µg/mL)	Spiked leachables*	Spiked Formalin (ppm)	Mono. ELISA (AU/mL)	Poly. ELISA (AU/mL)	Ratio	Mono. ELISA (AU/mL)	Poly. ELISA (AU/mL)	Ratio
1	7	100	50	1.4	0	9.725	12.814	0.76	6.342	3.649	0.575
2	7	0	0	1.4	0	15.254	16.331	0.93	9.865	8.039	0.815
3	7	0	0	0	40	12.457	12.614	0.99	9.037	8.176	0.905
4	8	0	50	1.4	0	13.513	12.971	1.04	10.328	9.533	0.923
5	7.5	50	50	0	0	13.592	14.924	0.91	10.358	8.032	0.775
6	7	0	50	0	40	12.942	13.878	0.93	10.008	9.896	0.989
7	7	100	50	0	0	9.649	12.608	0.77	6.089	4.092	0.672
8	8	0	50	0	0	11.184	11.902	0.94	9.113	9.048	0.993
9	8	100	0	0	0	11.436	12.883	0.89	8.858	7.409	0.836
10	8	100	50	0	0	13.361	15.516	0.86	10.525	8.158	0.775
11	8	0	50	1.4	40	13.209	13.608	0.97	9.349	9.201	0.984
12	7	100	0	1.4	0	8.4	11.913	0.71	5.001	2.951	0.590
13	8	0	0	0	40	10.294	10.483	0.98	8.019	8.284	1.033
14	7	100	0	0	40	9.972	12.015	0.83	7.435	5.802	0.780
15	8	0	0	1.4	0	14.096	15.618	0.9	10.192	9.531	0.935

Table 4 continued

16	8	100	50	0	40	9.78	13.514	0.72	11.011	8.947	0.813
17	7	0	50	1.4	0	11.213	14.285	0.78	10.246	8.377	0.818
18	7	0	50	1.4	40	11.183	11.499	0.97	10.539	9.217	0.875
19	8	100	0	1.4	40	10.536	10.935	0.96	10.151	8.341	0.822
20	8	0	0	1.4	40	10.026	9.654	1.04	11.306	9.281	0.821
21	8	0	50	0	40	10.149	9.986	1.02	11.213	9.205	0.821
22	8	100	0	1.4	0	10.051	12.193	0.82	10.918	6.841	0.627
23	7	0	0	1.4	40	10.024	10.74	0.93	10.711	7.963	0.743
24	7	100	50	0	40	9.535	10.254	0.93	10.642	6.991	0.657
25	7	0	50	0	0	11.143	11.765	0.95	13.054	8.684	0.665
26	7	0	0	0	0	11.431	11.796	0.97	13.753	10.391	0.756
27	8	100	50	1.4	0	10.09	11.953	0.84	11.506	7.334	0.637
28	8	100	0	0	40	10.137	11.223	0.9	11	8.131	0.739
29	8	100	50	1.4	40	9.605	10.535	0.91	10.848	7.939	0.732
30	7	100	0	0	0	6.485	8.379	0.77	7.957	3.523	0.443
31	7.5	50	0	0	0	11.081	12.229	0.91	12.377	9.535	0.770
32	8	0	0	0	0	11.421	12.002	0.95	11.36	9.859	0.868
33	7	100	50	1.4	40	9.03	10.583	0.85	9.753	6.571	0.674
34	7	100	0	1.4	40	9.05	10.927	0.83	11.715	6.872	0.587

Table 5: Analysis of Variance for ratio Monoclonal/Polyclonal ELISA after storage 4 weeks at 22°C

Analysis of Variance for Ratio 4 weeks

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:pH	0.02205	1	0.02205	5.48	0.0326
B:Alum	0.117612	1	0.117612	29.20	0.0001
C:PS Fragments	0.0008	1	0.0008	0.20	0.6618
D:Extractables	0.0008	1	0.0008	0.20	0.6618
E:Formaline	0.0242	1	0.0242	6.01	0.0261
AB	0.0001125	1	0.0001125	0.03	0.8694
AC	0.00045	1	0.00045	0.11	0.7425
AD	0.01125	1	0.01125	2.79	0.1141
AE	0.00405	1	0.00405	1.01	0.3309
BC	0.0000125	1	0.0000125	0.00	0.9563
BD	0.0010125	1	0.0010125	0.25	0.6229
BE	0.0006125	1	0.0006125	0.15	0.7017
CD	0.0008	1	0.0008	0.20	0.6618
CE	0.0008	1	0.0008	0.20	0.6618
DE	0.0072	1	0.0072	1.79	0.1999
Total error	0.0644375	16	0.00402734		
Total (corr.)	0.2562	31			

R-squared = 74.8488 percent
R-squared (adjusted for d.f.) = 51.2695 percent
Standard Error of Est. = 0.0634614
Mean absolute error = 0.0352734
Durbin-Watson statistic = 1.47556

Table 6: Analysis of Variance for ratio Monoclonal/Polyclonal ELISA after storage at 22°C for 8 weeks.

Analysis of Variance for Ratio 8 weeks

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:pH	0.102378	1	0.102378	11.19	0.0041
B:Alum	0.275653	1	0.275653	30.13	0.0000
C:PS Fragments	0.00300312	1	0.00300312	0.33	0.5747
D:Extractables	0.0108781	1	0.0108781	1.19	0.2917
E:Formaline	0.0318781	1	0.0318781	3.48	0.0804
AB	0.00137813	1	0.00137813	0.15	0.7031
AC	0.00382812	1	0.00382812	0.42	0.5269
AD	0.00137813	1	0.00137813	0.15	0.7031
AE	0.0166531	1	0.0166531	1.82	0.1961
BC	0.000253125	1	0.000253125	0.03	0.8700
BD	0.00382813	1	0.00382813	0.42	0.5269
BE	0.00195313	1	0.00195313	0.21	0.6503
CD	0.00195313	1	0.00195313	0.21	0.6503
CE	0.000153125	1	0.000153125	0.02	0.8987
DE	0.00525313	1	0.00525313	0.57	0.4596
Total error	0.1464	16	0.00915		
Total (corr.)	0.606822	31			

R-squared = 75.8743 percent
R-squared (adjusted for d.f.) = 53.2565 percent
Standard Error of Est. = 0.0956556
Mean absolute error = 0.0571484
Durbin-Watson statistic = 0.888586

Table 7: Regression analysis for "Ratio 8 weeks" including pH, Alum and Formaldehyde.

```
Regression coeffs. for Ratio 8 weeks
----------------------------------------------------------------------
constant     = 0.0228125
A:pH                              = 0.113125
B:Alum                            = -0.00185625
E:Formaline                       = 0.0315625
----------------------------------------------------------------------
```

Table 8: Estimation of results "Ratio 8 weeks" generated using the fitted model.

Estimation Results for Ratio 8 weeks

Row	Observed Value	Fitted Value	Lower 95.0% CL for Mean	Upper 95.0% CL for Mean
1	0.58	0.5975	0.536766	0.658234
2	0.81	0.783125	0.722391	0.843859
3	0.9	0.84625	0.785516	0.906984
4	0.92	0.89625	0.835516	0.956984
6	0.99	0.84625	0.785516	0.906984
7	0.67	0.5975	0.536766	0.658234
8	0.99	0.89625	0.835516	0.956984
9	0.84	0.710625	0.649891	0.771359
10	0.78	0.710625	0.649891	0.771359
11	0.98	0.959375	0.898641	1.02011
12	0.59	0.5975	0.536766	0.658234
13	1.03	0.959375	0.898641	1.02011
14	0.78	0.660625	0.599891	0.721359
15	0.94	0.89625	0.835516	0.956984
16	0.81	0.77375	0.713016	0.834484
17	0.82	0.783125	0.722391	0.843859
18	0.88	0.84625	0.785516	0.906984
19	0.82	0.77375	0.713016	0.834484
20	0.82	0.959375	0.898641	1.02011
21	0.82	0.959375	0.898641	1.02011
22	0.63	0.710625	0.649891	0.771359
23	0.74	0.84625	0.785516	0.906984
24	0.66	0.660625	0.599891	0.721359
25	0.67	0.783125	0.722391	0.843859
26	0.76	0.783125	0.722391	0.843859
27	0.64	0.710625	0.649891	0.771359
28	0.74	0.77375	0.713016	0.834484
29	0.73	0.77375	0.713016	0.834484
30	0.44	0.5975	0.536766	0.658234
32	0.87	0.89625	0.835516	0.956984
33	0.67	0.660625	0.599891	0.721359
34	0.59	0.660625	0.599891	0.721359

Table 9: Plan for experiment 20110913(NIV)

Pipetting plan						
			Stock Sol [mM]			
Total Volume:	2000 µl		Ni(II)SO4	1		
NIV material:	11A74		Cu(II)Cl2	1		
			Cr(III)Cl	1		

Sample			µg/L			
No.	Name	pH	Ni(II)SO4	Cu(II)Cl2	Cr(III)	fragmented PS
1	NIV_unspiked_pH7_22°C	7				
2	NIV_Ni(II)_100_pH7_22°C	7	100			
3	NIV_Ni(II)_500_pH7_22°C	7	500			
4	NIV_Ni(II)_1000_pH7_22°C	7	1000			
5	NIV_Cu(II)_100_pH7_22°C	7		100		
6	NIV_Cu(II)_500_pH7_22°C	7		500		
7	NIV_Cu(II)_1000_pH7_22°C	7		1000		
8	NIV_CR(III)_100_pH7_22°C	7			100	
9	NIV_CR(III)_500_pH7_22°C	7			500	
10	NIV_CR(III)_1000_pH7_22°C	7			1000	
11	NIV_PS spike_pH7_22°C	7				50
12	NIV_Ni(II)_100_PSspike_pH7_22°C	7	100			50
13	NIV_Ni(II)_500_PSspike_pH7_22°C	7	500			50
14	NIV_Ni(II)_1000_PSspike_pH7_22°C	7	1000			50
15	NIV_Cu(II)_100_PSspike_pH7_22°C	7		100		50

Table 9 continued

16	NIV_Cu(II)_500_PSspike_pH7_22℃	7		500		50
17	NIV_Cu(II)_1000_PSspike_pH7_22℃	7		1000		50
18	NIV_CR(III)_100_PSspike_pH7_22℃	7			100	50
19	NIV_CR(III)_500_PSspike_pH7_22℃	7			500	50
20	NIV_CR(III)_1000_PSspike_pH7_22℃	7			1000	50
21	NIV_unspiked_pH8_22℃	8				
22	NIV_Ni(II)_100_pH8_22℃	8	100			
23	NIV_Ni(II)_500_pH8_22℃	8	500			
24	NIV_Ni(II)_1000_pH8_22℃	8	1000			
25	NIV_Cu(II)_100_pH8_22℃	8		100		
26	NIV_Cu(II)_500_pH8_22℃	8		500		
27	NIV_Cu(II)_1000_pH8_22℃	8		1000		
28	NIV_CR(III)_100_pH8_22℃	8			100	
29	NIV_CR(III)_500_pH8_22℃	8			500	
30	NIV_CR(III)_1000_pH8_22℃	8			1000	
31	NIV_PS spike_pH8_22℃	8				50
32	NIV_Ni(II)_100_PSspike_pH8_22℃	8	100			50
33	NIV_Ni(II)_500_PSspike_pH8_22℃	8	500			50
34	NIV_Ni(II)_1000_PSspike_pH8_22℃	8	1000			50
35	NIV_Cu(II)_100_PSspike_pH8_22℃	8		100		50
36	NIV_Cu(II)_500_PSspike_pH8_22℃	8		500		50
37	NIV_Cu(II)_1000_PSspike_pH8_22℃	8		1000		50
38	NIV_CR(III)_100_PSspike_pH8_22℃	8			100	50
39	NIV_CR(III)_500_PSspike_pH8_22℃	8			500	50
40	NIV_CR(III)_1000_PSspike_pH8_22℃	8			1000	50

Table 9 continued

	MW [g/Mol]		other additives:						
Ni	58.7		Stock Sol:						
Cu	63.6		frag PS	2	mg/mL				
Cr	52.0								

Sample			Volume [µl]						
No.	Name	pH	NIV	PBS	Ni(II)SO4	Cu(II)Cl2	Cr(III)	fragmented PS	Total
1	NIV_unspiked_pH7_22℃	7	1800	200	0	0	0	0	2000
2	NIV_Ni(II)_100_pH7_22℃	7	1800	197	3	0	0	0	2000
3	NIV_Ni(II)_500_pH7_22℃	7	1800	183	17	0	0	0	2000
4	NIV_Ni(II)_1000_pH7_22℃	7	1800	166	34	0	0	0	2000
5	NIV_Cu(II)_100_pH7_22℃	7	1800	197	0	3	0	0	2000
6	NIV_Cu(II)_500_pH7_22℃	7	1800	184	0	16	0	0	2000
7	NIV_Cu(II)_1000_pH7_22℃	7	1800	169	0	31	0	0	2000
8	NIV_CR(III)_100_pH7_22℃	7	1800	196	0	0	4	0	2000
9	NIV_CR(III)_500_pH7_22℃	7	1800	181	0	0	19	0	2000
10	NIV_CR(III)_1000_pH7_22℃	7	1800	162	0	0	38	0	2000
11	NIV_PS spike_pH7_22℃	7	1800	150	0	0	0	50	2000
12	NIV_Ni(II)_100_PSspike_pH7_22℃	7	1800	147	3	0	0	50	2000
13	NIV_Ni(II)_500_PSspike_pH7_22℃	7	1800	133	17	0	0	50	2000
14	NIV_Ni(II)_1000_PSspike_pH7_22℃	7	1800	116	34	0	0	50	2000
15	NIV_Cu(II)_100_PSspike_pH7_22℃	7	1800	147	0	3	0	50	2000

Table 9 continued

16	NIV_Cu(II)_500_PSspike_pH7_22℃	7	1800	134	0	16	0	50	2000
17	NIV_Cu(II)_1000_PSspike_pH7_22℃	7	1800	119	0	31	0	50	2000
18	NIV_CR(III)_100_PSspike_pH7_22℃	7	1800	146	0	0	4	50	2000
19	NIV_CR(III)_500_PSspike_pH7_22℃	7	1800	131	0	0	19	50	2000
20	NIV_CR(III)_1000_PSspike_pH7_22℃	7	1800	112	0	0	38	50	2000
21	NIV_unspiked_pH8_22℃	8	1800	200	0	0	0	0	2000
22	NIV_Ni(II)_100_pH8_22℃	8	1800	197	3	0	0	0	2000
23	NIV_Ni(II)_500_pH8_22℃	8	1800	183	17	0	0	0	2000
24	NIV_Ni(II)_1000_pH8_22℃	8	1800	166	34	0	0	0	2000
25	NIV_Cu(II)_100_pH8_22℃	8	1800	197	0	3	0	0	2000
26	NIV_Cu(II)_500_pH8_22℃	8	1800	184	0	16	0	0	2000
27	NIV_Cu(II)_1000_pH8_22℃	8	1800	169	0	31	0	0	2000
28	NIV_CR(III)_100_pH8_22℃	8	1800	196	0	0	4	0	2000
29	NIV_CR(III)_500_pH8_22℃	8	1800	181	0	0	19	0	2000
30	NIV_CR(III)_1000_pH8_22℃	8	1800	162	0	0	38	0	2000
31	NIV_PS spike_pH8_22℃	8	1800	150	0	0	0	50	2000
32	NIV_Ni(II)_100_PSspike_pH8_22℃	8	1800	147	3	0	0	50	2000
33	NIV_Ni(II)_500_PSspike_pH8_22℃	8	1800	133	17	0	0	50	2000
34	NIV_Ni(II)_1000_PSspike_pH8_22℃	8	1800	116	34	0	0	50	2000
35	NIV_Cu(II)_100_PSspike_pH8_22℃	8	1800	147	0	3	0	50	2000
36	NIV_Cu(II)_500_PSspike_pH8_22℃	8	1800	134	0	16	0	50	2000
37	NIV_Cu(II)_1000_PSspike_pH8_22℃	8	1800	119	0	31	0	50	2000
38	NIV_CR(III)_100_PSspike_pH8_22℃	8	1800	146	0	0	4	50	2000
39	NIV_CR(III)_500_PSspike_pH8_22℃	8	1800	131	0	0	19	50	2000
40	NIV_CR(III)_1000_PSspike_pH8_22℃	8	1800	112	0	0	38	50	2000

Table 10: Plan for experiment 20110913(DP)

Pipetting plan

			Stock Sol [mM]	
Total Volume:	10000 µl	Ni(II)SO4	1	
		Cu(II)Cl2	1	
DP:	11D87 bulk	Cr(III)Cl3	1	

Sample			µg/L			
No.	Name	pH	Ni(II)SO4	Cu(II)Cl2	Cr(III)	fragmented PS
1	DP_unspiked_pH7	7				
2	DP_Ni(II)_100_pH7	7	100			
3	DP_Ni(II)_500_pH7	7	500			
4	DP_Ni(II)_1000_pH7	7	1000			
5	DP_Cu(II)_100_pH7	7		100		
6	DP_Cu(II)_500_pH7	7		500		
7	DP_Cu(II)_1000_pH7	7		1000		
8	DP_Cr(III)_100_pH7	7			100	
9	DP_Cr(III)_500_pH7	7			500	
10	DP_Cr(III)_1000_pH7	7			1000	
11	DP_unspiked_pH8	8				
12	DP_Ni(II)_100_pH8	8	100			
13	DP_Ni(II)_500_pH8	8	500			
14	DP_Ni(II)_1000_pH8	8	1000			
15	DP_Cu(II)_100_pH8	8		100		
16	DP_Cu(II)_500_pH8	8		500		
17	DP_Cu(II)_1000_pH8	8		1000		
18	DP_Cr(III)_100_pH8	8			100	
19	DP_Cr(III)_500_pH8	8			500	
20	DP_Cr(III)_1000_pH8	8			1000	

750

Table 10 continued

MW [g/Mol]			other additives:						
Ni	58.7		Stock Sol:						
Cu	63.6		frag PS	2	mg/mL				
Cr	52.0								

Sample			Volume [µl]						
No.	Name	pH	DP	Buffer	Ni(II)SO4	Cu(II)Cl2	Cr(III)	fragmented PS	Total
1	DP_unspiked_pH7	7	9500	500	0	0	0	0	10000
2	DP_Ni(II)_100_pH7	7	9500	483	17	0	0	0	10000
3	DP_Ni(II)_500_pH7	7	9500	415	85	0	0	0	10000
4	DP_Ni(II)_1000_pH7	7	9500	330	170	0	0	0	10000
5	DP_Cu(II)_100_pH7	7	9500	484	0	16	0	0	10000
6	DP_Cu(II)_500_pH7	7	9500	421	0	79	0	0	10000
7	DP_Cu(II)_1000_pH7	7	9500	343	0	157	0	0	10000
8	DP_Cr(III)_100_pH7	7	9500	481	0	0	19	0	10000
9	DP_Cr(III)_500_pH7	7	9500	404	0	0	96	0	10000
10	DP_Cr(III)_1000_pH7	7	9500	308	0	0	192	0	10000
11	DP_unspiked_pH8	8	9500	500	0	0	0	0	10000
12	DP_Ni(II)_100_pH8	8	9500	483	17	0	0	0	10000
13	DP_Ni(II)_500_pH8	8	9500	415	85	0	0	0	10000
14	DP_Ni(II)_1000_pH8	8	9500	330	170	0	0	0	10000
15	DP_Cu(II)_100_pH8	8	9500	484	0	16	0	0	10000
16	DP_Cu(II)_500_pH8	8	9500	421	0	79	0	0	10000
17	DP_Cu(II)_1000_pH8	8	9500	343	0	157	0	0	10000
18	DP_Cr(III)_100_pH8	8	9500	481	0	0	19	0	10000
19	DP_Cr(III)_500_pH8	8	9500	404	0	0	96	0	10000
20	DP_Cr(III)_1000_pH8	8	9500	308	0	0	192	0	10000

Table 11: Plan for experiment 20110812-Metal Ion Spiked DP

Pipetting plan										
			Stock Sol [mM]			MW [g/Mol]		FVL ng/mL	µM
Total Volume:	30000 µl		Fe(II)Cl3	1		Fe	55.9	282	5.049
DP:	11D87 bulk		Fe(III)Cl3	1		Fe	55.9	282	5.045
			Ni(II)SO4	1		Ni	58.7	41	0.698
			Co(II)Cl2	1		Co	58.9	0.33	0.006
			Cu(II)Cl2	1		Cu	63.6	3	0.047
			Zn(II)SO4	1		Zn	65.4		
			Cr(III)Cl3	1		Cr	52.0		

Sample			µg/L						
No.	Name	pH	DP dil	Fe(II)Cl3	Fe(III)Cl3	Ni(II)SO4	Co(II)Cl2	Cu(II)Cl2	Zn(II)SO4
1	DP_Fe(II)_pH7	7	1	500					
2	DP_Fe(III)_pH7	7	1		500				
3	DP_Ni(II)_pH7	7	1			500			
4	DP_Co(II)_pH7	7	1				500		
5	DP_Cu(II)_pH7	7	1					500	
6	DP_Zn_pH7	7	1						500
7	DP_metalmix_pH7	7	1	500	500	500	500	500	500
8	DP_unspiked_pH7	7	1						
9	DP_Fe(II)_pH7.4	7.4	1	500					
10	DP_Fe(III)_pH7.4	7.4	1		500				
11	DP_Ni(II)_pH7.4	7.4	1			500			
12	DP_Co(II)_pH7.4	7.4	1				500		
13	DP_Cu(II)_pH7.4	7.4	1					500	
14	DP_Zn_pH7.4	7.4	1						500
15	DP_metalmix_pH7.4	7.4	1	500	500	500	500	500	500
16	DP_unspiked_pH7.4	7.4	1						
17	DP_Fe(II)_pH7.8	7.8	1	500					
18	DP_Fe(III)_pH7.8	7.8	1		500				
19	DP_Ni(II)_pH7.8	7.8	1			500			
20	DP_Co(II)_pH7.8	7.8	1				500		
21	DP_Cu(II)_pH7.8	7.8	1					500	
22	DP_Zn_pH7.8	7.8	1						500
23	DP_metalmix_pH7.8	7.8	1	500	500	500	500	500	500
24	DP_unspiked_pH7.8	7.8	1						

Table 11 continued

Sample			µg/L							
No.	Name	pH	DP dil	Cr(III)						
25	DP_CR(III)_pH7	7	1	500						
26	DP_Cr(III)_pH7.4	7.4	1	500						
27	DP_Cr(III)_pH7.8	7.8	1	500						

Sample			Volume [µl]								
No.	Name	pH	NIV dil	Buffer	Fe(II)Cl3	Fe(III)Cl3	Ni(II)SO4	Co(II)Cl2	Cu(II)Cl2	Zn(II)SO4	Total
1	DP_Fe(II)_pH7	7	27750	1981	269	0	0	0	0	0	30000
2	DP_Fe(III)_pH7	7	27750	1982	0	268	0	0	0	0	30000
3	DP_Ni(II)_pH7	7	27750	1981	0	0	269	0	0	0	30000
4	DP_Co(II)_pH7	7	27750	1994	0	0	0	256	0	0	30000
5	DP_Cu(II)_pH7	7	27750	1995	0	0	0	0	255	0	30000
6	DP_Zn_pH7	7	27750	2014	0	0	0	0	0	236	30000
7	DP_metalmix_pH7	7	27750	698	269	268	269	256	255	236	30000
8	DP_unspiked_pH7	7	27750	2250	0	0	0	0	0	0	30000
9	DP_Fe(II)_pH7.4	7.4	27750	1981	269	0	0	0	0	0	30000
10	DP_Fe(III)_pH7.4	7.4	27750	1982	0	268	0	0	0	0	30000
11	DP_Ni(II)_pH7.4	7.4	27750	1981	0	0	269	0	0	0	30000
12	DP_Co(II)_pH7.4	7.4	27750	1994	0	0	0	256	0	0	30000
13	DP_Cu(II)_pH7.4	7.4	27750	1995	0	0	0	0	255	0	30000
14	DP_Zn_pH7.4	7.4	27750	2014	0	0	0	0	0	236	30000
15	DP_metalmix_pH7.4	7.4	27750	698	269	268	269	256	255	236	30000
16	DP_unspiked_pH7.4	7.4	27750	2250	0	0	0	0	0	0	30000
17	DP_Fe(II)_pH7.8	7.8	27750	1981	269	0	0	0	0	0	30000
18	DP_Fe(III)_pH7.8	7.8	27750	1982	0	268	0	0	0	0	30000
19	DP_Ni(II)_pH7.8	7.8	27750	1981	0	0	269	0	0	0	30000
20	DP_Co(II)_pH7.8	7.8	27750	1994	0	0	0	256	0	0	30000
21	DP_Cu(II)_pH7.8	7.8	27750	1995	0	0	0	0	255	0	30000
22	DP_Zn_pH7.8	7.8	27750	2014	0	0	0	0	0	236	30000
23	DP_metalmix_pH7.8	7.8	27750	698	269	268	269	256	255	236	30000
24	DP_unspiked_pH7.8	7.8	27750	2250	0	0	0	0	0	0	30000

Table 11 continued

Sample			Volume [µl]							
No.	Name	pH			Cr(III)Cl3					
8	DP_CR(III)_pH7	7			48					
16	DP_Cr(III)_pH7.4	7.4			48					
24	DP_Cr(III)_pH7.8	7.8			48					

Table 12: Antigen recovery determined by SEC-HPLC of exp. 20110913(NIV). For pH 7 and pH 8 the recoveries are based on non-spiked NIV control samples #1 and #21, respectively. Samples were stored at 22°C for 3 weeks. Samples marked with "n.a" were not analyzed due to sample prioritization

		Results	
Sample		Area mAU*s	Recovery (%)
1	NIV_unspiked_pH7_22°C	4.054	100.0
2	NIV_Ni(II)_100_pH7_22°C	3.972	98.0
3	NIV_Ni(II)_500_pH7_22°C	4.454	109.9
4	NIV_Ni(II)_1000_pH7_22°C	4.185	103.2
5	NIV_Cu(II)_100_pH7_22°C	3.913	96.5
6	NIV_Cu(II)_500_pH7_22°C	3.733	92.1
7	NIV_Cu(II)_1000_pH7_22°C	3.115	76.8
8	NIV_CR(III)_100_pH7_22°C	3.957	97.6
9	NIV_CR(III)_500_pH7_22°C	4.000	98.7
10	NIV_CR(III)_1000_pH7_22°C	3.611	89.1
11	NIV_PS spike_pH7_22°C	4.068	100.3
12	NIV_Ni(II)_100_PSspike_pH7_22°C	3.884	95.8
13	NIV_Ni(II)_500_PSspike_pH7_22°C	3.688	91.0
14	NIV_Ni(II)_1000_PSspike_pH7_22°C	3.971	98.0
15	NIV_Cu(II)_100_PSspike_pH7_22°C	3.568	88.0
16	NIV_Cu(II)_500_PSspike_pH7_22°C	3.325	82.0
17	NIV_Cu(II)_1000_PSspike_pH7_22°C	3.486	86.0
18	NIV_CR(III)_100_PSspike_pH7_22°C	3.747	92.4
19	NIV_CR(III)_500_PSspike_pH7_22°C	3.904	96.3
20	NIV_CR(III)_1000_PSspike_pH7_22°C	3.685	90.9
21	NIV_unspiked_pH8_22°C	4.213	100.0
22	NIV_Ni(II)_100_pH8_22°C	4.181	99.2
23	NIV_Ni(II)_500_pH8_22°C	4.150	98.5
24	NIV_Ni(II)_1000_pH8_22°C	3.772	89.5
25	NIV_Cu(II)_100_pH8_22°C	4.146	98.4
26	NIV_Cu(II)_500_pH8_22°C	4.212	100.0
27	NIV_Cu(II)_1000_pH8_22°C	4.152	98.6
28	NIV_CR(III)_100_pH8_22°C	4.213	100.0
29	NIV_CR(III)_500_pH8_22°C	3.997	94.9

30	NIV_CR(III)_1000_pH8_22°C	4.231	100.4
31	NIV_PS spike_pH8_22°C	4.150	98.5
32	NIV_Ni(II)_100_PSspike_pH8_22°C	3.623	86.0
33	NIV_Ni(II)_500_PSspike_pH8_22°C	3.725	88.4
34	NIV_Ni(II)_1000_PSspike_pH8_22°C	4.079	96.8
35	NIV_Cu(II)_100_PSspike_pH8_22°C	3.473	82.4
36	NIV_Cu(II)_500_PSspike_pH8_22°C	3.180	75.5
37	NIV_Cu(II)_1000_PSspike_pH8_22°C	4.056	96.3
38	NIV_CR(III)_100_PSspike_pH8_22°C	3.042	72.2
39	NIV_CR(III)_500_PSspike_pH8_22°C	4.113	97.6
40	NIV_CR(III)_1000_PSspike_pH8_22°C	n.a.	n.a.

Table 13: ELISA results of exp. 20110913(NIV) obtained after 3 weeks at pH 8 (sample 21-40) and 7 weeks at pH 7(sample 1-20) at 22°C. Samples marked with "n.a" were not analyzed due to sample prioritization.

20110913_metal spiked_NIV_22°C 7 weeks at pH 7				
No.	**Name**	**poly**	**mono**	**ratio**
1	NIV_unspiked_pH7_22°C CONTROL	20.699	17.512	0.846
2	NIV_Ni(II)_100_pH7_22°C	17.243	14.787	0.858
3	NIV_Ni(II)_500_pH7_22°C	18.877	16.215	0.859
4	NIV_Ni(II)_1000_pH7_22°C	16.9	14.522	0.859
5	NIV_Cu(II)_100_pH7_22°C	16.718	13.278	0.794
6	NIV_Cu(II)_500_pH7_22°C	14.664	5.459	0.372
7	NIV_Cu(II)_1000_pH7_22°C	7.112	0.421	0.059
8	NIV_CR(III)_100_pH7_22°C	19.207	16.602	0.864
9	NIV_CR(III)_500_pH7_22°C	20.313	17.84	0.878
10	NIV_CR(III)_1000_pH7_22°C	16.762	13.128	0.783
11	NIV_PS spike_pH7_22°C CONTROL	19.907	16.994	0.854
12	NIV_Ni(II)_100_PSspike_pH7_22°C	19.546	16.534	0.846
13	NIV_Ni(II)_500_PSspike_pH7_22°C	18.337	15.2	0.829
14	NIV_Ni(II)_1000_PSspike_pH7_22°C	20.759	16.934	0.816
15	NIV_Cu(II)_100_PSspike_pH7_22°C	18.249	13.348	0.731
16	NIV_Cu(II)_500_PSspike_pH7_22°C	19.201	7.733	0.403
17	NIV_Cu(II)_1000_PSspike_pH7_22°C	8.515	0.711	0.083
18	NIV_CR(III)_100_PSspike_pH7_22°C	18.377	16.521	0.899
19	NIV_CR(III)_500_PSspike_pH7_22°C	19.678	17.119	0.870
20	NIV_CR(III)_1000_PSspike_pH7_22°C	20.505	18.219	0.889

20110913_metal spiked_NIV_22°C 3 weeks at pH 8							
		1st analysis			2nd analysis		
No		poly	mono	ratio	poly	mono	ratio
21	NIV_unspiked_pH8_22°C CONTROL	22.729	20.679	0.910	24.687	24.806	1.005
22	NIV_Ni(II)_100_pH8_22°C	23.732	23.572	0.993	24.179	22.148	0.916
23	NIV_Ni(II)_500_pH8_22°C	20.086	19.793	0.985	22.411	22.207	0.991
24	NIV_Ni(II)_1000_pH8_22°C	16.553	15.402	0.930	23.841	21.645	0.908
25	NIV_Cu(II)_100_pH8_22°C	18.736	18.175	0.970	28.024	24.714	0.882
26	NIV_Cu(II)_500_pH8_22°C	21.173	19.109	0.903	25.774	23.983	0.931
27	NIV_Cu(II)_1000_pH8_22°C	19.709	16.406	0.832	24.799	21.580	0.870
28	NIV_CR(III)_100_pH8_22°C	22.464	20.687	0.921	22.782	21.156	0.929
29	NIV_CR(III)_500_pH8_22°C	20.527	20.247	0.986	23.040	21.649	0.940
30	NIV_CR(III)_1000_pH8_22°C	20.838	19.094	0.916	25.676	24.047	0.937
31	NIV_PS spike_pH8_22°C CONTROL	19.051	20.112	1.056	25.413	25.991	1.023
32	NIV_Ni(II)_100_PSspike_pH8_22°C	18.729	19.250	1.028	n.a.	n.a.	n.a.
33	NIV_Ni(II)_500_PSspike_pH8_22°C	20.923	20.516	0.981	n.a.	n.a.	n.a.
34	NIV_Ni(II)_1000_PSspike_pH8_22°C	21.734	19.794	0.911	25.164	24.899	0.989
35	NIV_Cu(II)_100_PSspike_pH8_22°C	21.526	19.852	0.922	n.a.	n.a.	n.a.
36	NIV_Cu(II)_500_PSspike_pH8_22°C	21.914	19.449	0.888	n.a.	n.a.	n.a.
37	NIV_Cu(II)_1000_PSspike_pH8_22°C	18.646	16.259	0.872	25.371	24.275	0.957
38	NIV_CR(III)_100_PSspike_pH8_22°C	20.292	18.667	0.920	n.a.	n.a.	n.a.
39	NIV_CR(III)_500_PSspike_pH8_22°C	22.835	21.558	0.944	25.561	24.480	0.958
40	NIV_CR(III)_1000_PSspike_pH8_22°C	27.862	25.291	0.908	n.a.	n.a.	n.a.

Table 14: Antigen recoveries after 5 weeks at 22°C of desorbed JEV obtained by SEC-HPLC. Recoveries were based on non-spiked DP control samples stored at either pH 7 or pH 8

		JEV area	
No	**Sample**	**mAU.min**	**recovery**
1	DP_unspiked_pH7 22°C	3.710	100%
2	DP_Ni(II)_100_pH7 22°C	3.656	99%
3	DP_Ni(II)_500_pH7 22°C	3.705	100%
4	DP_Ni(II)_1000_pH7 22°C	3.444	93%
5	DP_Cu(II)_100_pH7 22°C	3.565	96%
6	DP_Cu(II)_500_pH7 22°C	3.313	89%
7	DP_Cu(II)_1000_pH7 22°C	3.367	91%
8	DP_Cr(III)_100_pH7 22°C	3.562	96%

9	DP_Cr(III)_500_pH7 22°C	3.422	92%
10	DP_Cr(III)_1000_pH7 22°C	3.148	85%
11	DP_unspiked_pH8 22°C	4.297	100%
12	DP_Ni(II)_100_pH8 22°C	4.029	94%
13	DP_Ni(II)_500_pH8 22°C	4.306	100%
14	DP_Ni(II)_1000_pH8 22°C	4.065	95%
15	DP_Cu(II)_100_pH8 22°C	3.751	87%
16	DP_Cu(II)_500_pH8 22°C	3.698	86%
17	DP_Cu(II)_1000_pH8 22°C	3.511	82%
18	DP_Cr(III)_100_pH8 22°C	3.805	89%
19	DP_Cr(III)_500_pH8 22°C	3.843	89%
20	DP_Cr(III)_1000_pH8 22°C	4.212	98%

Table 15: ELISA results of desorbed JEV antigen after 5 weeks at 22°C

No.	Name	pH	Poly. ELISA	Mono. ELISA	Ratio	% Ratio compared to non-spiked Control
1	**DP_unspiked_pH7**	**7**	**14.203**	**13.13**	**0.924**	**100**
2	DP_Ni(II)_100_pH7	7	13.089	12.623	0.964	104
3	DP_Ni(II)_500_pH7	7	12.640	12.572	0.995	108
4	DP_Ni(II)_1000_pH7	7	15.051	11.757	0.781	84
5	DP_Cu(II)_100_pH7	7	13.420	10.792	0.804	87
6	DP_Cu(II)_500_pH7	7	13.247	10.079	0.761	82
7	DP_Cu(II)_1000_pH7	7	12.981	9.654	0.744	80
8	DP_Cr(III)_100_pH7	7	16.936	11.886	0.702	76
9	DP_Cr(III)_500_pH7	7	13.991	11.219	0.802	87
10	DP_Cr(III)_1000_pH7	7	13.061	10.438	0.799	86
11	**DP_unspiked_pH8**	**8**	**12.647**	**11.287**	**0.892**	**100**
12	DP_Ni(II)_100_pH8	8	12.308	10.689	0.868	97
13	DP_Ni(II)_500_pH8	8	14.300	12.623	0.883	99
14	DP_Ni(II)_1000_pH8	8	12.082	10.930	0.905	101
15	DP_Cu(II)_100_pH8	8	11.041	9.937	0.900	101
16	DP_Cu(II)_500_pH8	8	9.869	9.176	0.930	104
17	DP_Cu(II)_1000_pH8	8	9.379	8.802	0.938	105
18	DP_Cr(III)_100_pH8	8	10.164	9.545	0.939	105
19	DP_Cr(III)_500_pH8	8	11.241	10.057	0.895	100
20	DP_Cr(III)_1000_pH8	8	12.400	11.183	0.902	101

Table 16: ELISA results of desorbed JEV antigen after 4 weeks and 7 weeks stored at 22°C. Samples marked with "n.a" were not analyzed due to sample prioritization

			4 weeks @ 22°C			7 weeks @ 22°C		
No		**pH**	poly	mono	ratio	poly	mono	ratio m/p
1	DP Fe(II) pH7	7	14.285	11.213	0.785	14.181	11.378	0.802
2	DP Fe(III) pH7	7	14.879	11.552	0.776	14.323	11.765	0.821
3	DP Ni(II) pH7	7	16.572	11.862	0.716	14.231	11.666	0.820
4	DP Co(II) pH7	7	12.81	12.629	0.986	14.246	11.244	0.789
5	DP Cu(II) pH7	7	12.474	9.747	0.781	11.464	7.654	0.668
6	DP Zn(II) pH7	7	13.131	11.186	0.852	15.122	11.514	0.761
25	DP Cr(III) pH7	7	12.144	11.598	0.955	13.543	10.73	0.792
7	DP metalmix pH7	7	11.078	8.366	0.755	8.617	5.392	0.626
8	**DP unspiked pH7**	7	**16.159**	**13.224**	**0.818**	**14.158**	**11.559**	**0.816**
9	DP Fe(II) pH7.4	7.4	15.096	12.649	0.838	14.417	11.239	0.780
10	DP Fe(III) pH7.4	7.4	13.509	12.029	0.890	16.948	12.344	0.728
11	DP Ni(II) pH7.4	7.4	13.036	11.306	0.867	13.293	12.571	0.946
12	DP Co(II) pH7.4	7.4	13.715	11.714	0.854	13.079	11.96	0.914
13	DP Cu(II) pH7.4	7.4	14.235	11.748	0.825	10.843	9.276	0.855
14	DP Zn(II) pH7.4	7.4	13.815	12.882	0.932	13.28	12.619	0.950
26	DP Cr(III) pH7.4	7.4	12.324	11.659	0.946	13.312	10.804	0.812
15	DP_metalmix_pH7.4	7.4	n.a.			9.74	6.551	0.673
16	**DP unspiked pH7.4**	**7.4**	**13.951**	**13.225**	**0.948**	**11.97**	**12.672**	**1.059**
17	DP Fe(II) pH7.8	7.8	14.356	13.102	0.913	12.625	13.304	1.054
18	DP Fe(III) pH7.8	7.8	13.554	12.388	0.914	13.003	10.811	0.831
19	DP Ni(II) pH7.8	7.8	13.949	12.496	0.896	13.106	11.757	0.897
20	DP Co(II) pH7.8	7.8	12.826	11.931	0.930	13.333	11.059	0.829
21	DP Cu(II) pH7.8	7.8	12.593	11.268	0.895	12.538	9.872	0.787
22	DPZn(II) pH7.8	7.8	15.217	14.204	0.933	15.55	13.217	0.850
27	DP Cr(III) pH7.8	7.8	12.977	13.228	1.019	14.642	11.975	0.818
23	DP metalmix pH7.8	7.8	11.196	9.811	0.876	10.771	7.539	0.700
24	**DP unspiked pH7.8**	**7.8**	**11.906**	**11.819**	**0.993**	**12.472**	**11.034**	**0.885**

Table 17: ANOVA for stability samples stored at 22°C for 7 weeks.

Analysis of Variance for Ratio - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Metal Type	0.139643	8	0.0174554	3.00	0.0293
B:pH	0.0462028	2	0.0231014	3.97	0.0398
RESIDUAL	0.0931046	16	0.00581904		
TOTAL (CORRECTED)	0.27895	26			

All F-ratios are based on the residual mean square error.

Table 18: Multiple range test for ratio by metal ion type. 1=Fe(II); 2=Fe(III); 3=Ni(II); 4=Co(II); 5=Cu(II); 6=Zn(II); 7=Cr(III); 8=Mix[1-6]; 9=non-spiked control

Multiple Range Tests for Ratio by Metal Type

Method: 95.0 percent LSD

Metal Type	Count	LS Mean	Homogeneous Groups
8	3	0.666087	X
5	3	0.770168	XX
2	3	0.793725	XXX
7	3	0.807248	XX
4	3	0.844388	XX
6	3	0.853867	XX
1	3	0.878563	XX
3	3	0.887505	XX
9	3	0.919926	X

Contrast	Difference	+/- Limits
1 - 2	0.084838	0.132037
1 - 3	-0.0089421	0.132037
1 - 4	0.0341754	0.132037
1 - 5	0.108395	0.132037
1 - 6	0.0246961	0.132037
1 - 7	0.0713155	0.132037
1 - 8	*0.212476	0.132037
1 - 9	-0.0413627	0.132037
2 - 3	-0.0937801	0.132037
2 - 4	-0.0506626	0.132037
2 - 5	0.0235569	0.132037
2 - 6	-0.0601419	0.132037
2 - 7	-0.0135225	0.132037
2 - 8	0.127638	0.132037
2 - 9	-0.126201	0.132037
3 - 4	0.0431175	0.132037
3 - 5	0.117337	0.132037
3 - 6	0.0336382	0.132037
3 - 7	0.0802576	0.132037
3 - 8	*0.221418	0.132037
3 - 9	-0.0324206	0.132037
4 - 5	0.0742195	0.132037
4 - 6	-0.00947935	0.132037
4 - 7	0.0371401	0.132037
4 - 8	*0.1783	0.132037
4 - 9	-0.0755381	0.132037
5 - 6	-0.0836988	0.132037
5 - 7	-0.0370794	0.132037
5 - 8	0.104081	0.132037
5 - 9	*-0.149758	0.132037
6 - 7	0.0466195	0.132037
6 - 8	*0.18778	0.132037
6 - 9	-0.0660588	0.132037
7 - 8	*0.14116	0.132037
7 - 9	-0.112678	0.132037
8 - 9	*-0.253838	0.132037

* denotes a statistically significant difference.

Table 19: ICP-MS results of residual metal ion impurities present in various Alum (2%) lots

	Residual metal content (ng/mL)					
Alum (2%)Lot	**Cr**	**Fe**	**Ni**	**Cu**	**V**	**Co**
4074	19.8	266	14.8	<25	<5	<5
4470	1637	1179	17.5	<25	<5	<5
4563	1874	2485	8.9	<25	<5	<5
4621	1333	1183	7.6	<25	<5	<5
3877	48.2	183	12.2	<25	<5	<5
4230 (nonGI**, GI***)	1139	5640	816	64	12.6	7
Mix* 4074/4230	579.4	2953	415.4	<44.5	<6	<6

*Calculated content of residual metals based on Alum lot 4230 and 4074

**nonGI: non gamma irradiated

***GI: gamma irradiated

Table 20: Summary of metal ion content and analysis of DP samples formulated with various Alum lots. Samples were analysed in duplicate by ELISA and the ratio of monoclonal/polyclonal ELISA is reported. Formulations were stored at 22°C for 6 weeks.

#	Alum Lot (2% Stock solution)	Monoclonal 1st analysis	Polyclonal 1st analysis	Monoclonal 2nd analysis	Polyclonal 2nd analysis	Ratio 1st analysis	Ratio 2nd analysis	Mean ratio (Mono/poly)	Range* Ratio
1	4470	23.584	26.397	21.666	22.948	0.893	0.944	**0.918**	0.025
2	4563	23.027	23.397	19.051	18.862	0.984	1.010	**0.997**	0.013
3	4621	22.758	24.056	19.041	19.196	0.946	0.991	**0.968**	0.023
4	3877	23.5	23.85	21.186	21.24	0.985	0.997	**0.991**	0.006
5	4230 (non gamma irradiated)	21.85	25.155	20.682	23.792	0.868	0.869	**0.869**	0.000
6	4230 (gamma irradiated)	20.509	22.904	18.002	23.512	0.895	0.765	**0.830**	0.065
7	4074	23.022	24.047	16.695	19.866	0.957	0.840	**0.898**	0.058
8	Mixture (50%/50%) of 4074 and 4230	20.833	22.954	22.473	21.217	0.908	1.059	**0.983**	0.076

*Range is the absolute difference between 1st and 2nd analysis.

Table 21: overview of Alhydrogel® lots used in JEV production

lot #	Brenntag ID	Ammonia alum source	used for # of JEV batches
1	4074	BK Giulini	17
2	4230	Holland (USA)	24
3	4414	Canton India	7
4	4470	Canton India	19
5	4539	Holland (USA)	4

Table 22: Results of PSD analysis of Alhydrogel® (2%) stock solution in water.

No	Sample Name	d (0.1)	d (0.5)	d (0.9)	Obscuration (%)
1	Non-irradiated AlOH RQCS0890 Lot 4230	0.70	2.13	46.53	1.51
2	GI AlOH RQCS1200 Lot 4230	0.71	4.14	69.64	1.98
3	GI AlOH RQCS1342 Lot 4740	0.78	2.23	53.44	2.11
4	GI AlOH RQCS0448 Lot 4074	0.73	4.49	78.58	1.96

d(0.1): 10% of all measured particles have a diameter below this value

d(0.5): 50% of all measured particles have a diameter below this value

d(0.9): 90% of all measured particles have a diameter below this value

Obscuration: amount of laser light reduction by sample; corresponds to concentration of sample in measurement chamber

Table 23: Results of Alhydrogel® titration curves for determination of POZ. Samples were analyzed in PBS (1:20 dilution).

Sample ID	**PZC (pH)**
Non-irradiated AlOH RQCS0890 Lot 4230	4.58
GI AlOH RQCS1200 Lot 4230	4.62
GI AlOH RQCS1342 Lot 4740	4.49
GI AlOH RQCS0448 Lot 4074	4.48

Table 24: Summary of metal ion analysis for various Aluminum hydroxide stock solutions; Note: A 2% stock solution equals 10mg/mL of Al

Alhydrogel® (2% solution)	**Al**	**Fe**	**Ni**	**Cu**	**Co**	**Cr**	**Ag**	**Cd**	**W**	**Pb**	**V**	**Rb**	**Mo**
	µg/mL	**ng/mL**											
Lot 4074 (RQCS0013)	9130	266	15	<25	<5	20	<5	<5	<25	19	<5	<5	<5
Lot 4230 (RQCS 0890)	9570	5640	816	64	7	1139	<5	<5	<25	24	13	<5	11
Lot 4470 (RQCS1254)	9560	1179	18	<25	<5	1637	<5	<5	<25	20	<5	<5	22
Lot 4414 (RQCS1220)	10272	2790	36	<25	<5	1710	<5	<5	35	<25	<7	n.a.	n.a.
Lot 4539	n.a.	943	119	<25	<5	276	<5	<5	<25	27	< 5	<5	<5
Lot 3877	10766	183	12	<25	<5	48	<5	<5	<25	10	<5	<5	<5
Lot 4187	14100	3617	172	<25	<5	2333	<5	<5	<25	18	< 5	<5	<5
Lot 4287	9800	2047	296	<25	<5	620	<5	<5	<25	30	10	<5	<5
Lot 4563	9360	2485	9	<25	<5	1874	<5	<5	<25	8	< 5	<5	<5
Lot 4621	9760	1183	8	<25	<5	1333	<5	<5	<25	8	< 5	<5	<5
Lot 4580 (7xwashed)	10497	2610	27	<25	<5	1470	<5	<5	<25	<25	< 5	n.a.	n.a.
Lot 4596 (7xwashed)	10776	3530	27	<25	<5	1710	<5	<5	<25	<25	< 5	n.a.	n.a.
Lot 4577 (7xwashed)	10720	3060	24	<25	<5	1650	<5	<5	<25	<25	< 5	n.a.	n.a.
average	10359	2272	121	n.a.**	<5	1217	<5	<5	n.a.**	18*	11*	<5	16*
stdev	1312	1538	225			745				8	2		8
min	9130	183	8	<25	<5	20	<5	<5	<25	8	10	<5	<5
max	14100	5640	816	64	7	2333	<5	<5	35	30	13	<5	22
RSD (%)	**13**	**68**	**186**			**61**				**44**	**14**		**47**

*results below LOQ were not used for average calculation; **no average calculation possible due to results below LOQ

Table 25: Analysis of supernatant and Aluminum hydroxide (Lot 4230) gel fraction for contaminating metal ions shows metal ions are located in the gel, not the supernatant

Sample	**Fe**	**Ni**	**Cu**	**Co**	**Cr**	**Ag**	**Cd**	**W**	**Pb**	**V**
	ng/mL									
Lot 4230 Supernatant	82	12	<25	<5	7	<5	<5	<25	70	<5
Lot 4230 Sediment	6200	920	<25	<5	1200	<5	<5	<25	45	13
% supernatant compared to sediment	1.3	1.3	n.a.	n.a.	0.6	n.a.	n.a.	n.a.	155.6	n.a.

Table 26: Overview on raw materials used for Alhydrogel® production

Ammonia Alum lot	**Origin**	**Resulting Alhydrogel batch**	**# JEV production batches**
6-80578-28	BK Giulini	4074	**17** (e.g. JEV08A02)
AA0427		4230	**24** (e.g. JEV09**L**37)
91480	Canton India	4470	**19** (e.g. JEV10F54)
10094	*unknown*	*4563*	***Not used***
10095			

Table 27: Results of ICP-MS analysis for Ammonia Alum raw materials; lots in bold were used in JEV vaccine production

Ammonia Alum	**Used for Alhydrogel® Lot**	**Al**	**Fe**	**Ni**	**Cu**	**Co**	**Cr**	**Ag**	**Cd**	**W**	**Pb**	**V**
		mg/kg	**µg/kg**									
6-80578-28	**4074**	60400	770	16	<25	<10	60	<10	<10	<25	<25	<10
AA0427	**4230**	59800	25700	3560	210	25	6470	<10	15	<25	40	70
91480	**4470**	59800	8200	50	<25	<10	11700	<10	<10	<25	<25	16
10094	*4563*	*59800*	*16000*	*20*	*<25*	*<10*	*11200*	*<10*	*<10*	*<25*	*<25*	*20*
10095	*4563*	*60100*	*19400*	*20*	*<25*	*<10*	*13100*	*<10*	*<10*	*<25*	*<25*	*25*
			ng/mL									
24% Ammonia solution # 4140716			<2,0	1,5	28	<0,2	0,9	<0,2	<0,2	<1,0	<0,2	<0,2

Table 28: Comparison of Ammonia Alum and corresponding Alhydrogel® samples

		Fe	Cr	Ni	Cu
		rel %			
Ammonia Alum Lot	**6-80578**	88	7	2	3
Alum Lot	4074	82	6	5	8
Ammonia Alum Lot	**AA0427**	72	18	10	1
Alum Lot	4230	74	15	11	1
Ammonia Alum Lot	**91480**	41	59	0	0
Alum Lot	4470	41	57	1	1

Table 29: Summary of 70 element scan performed at ESG. Only those elements where differences were observed between individual lots are shown.

Alum 4074		Ammonia-Alum 6-80579-28
Laboratory Reference		ASC/ 2666.002
	LOD	mg/kg
Al	3	59000
Ca	0.8	<0.8
Cr	0.2	<0.2
Fe	0.3	0.4
K	3	5
Li	0.003	<0.003
Mg	0.05	<0.05
Mo	0.03	<0.03
Na	1	3
Ni	0.3	<0.3
Rb	0.02	<0.02
Zr	0.02	<0.02

Alum 4563		Ammonia-Alum 10094
Laboratory Reference		ASC/ 2666.003
	LOD	mg/kg
Al	3	59000
Ca	0.8	<0.8
Cr	0.2	4.9
Fe	0.3	18
K	3	200
Li	0.003	<0.003
Mg	0.05	1.6
Mo	0.03	<0.03
Na	1	<1
Ni	0.3	<0.3
Rb	0.02	1
Zr	0.02	<0.02

Alum 4563		Ammonia-Alum 10095
Laboratory Reference		ASC/ 2666.004
	LOD	mg/kg
Al	3	59000
Ca	0.8	<0.8
Cr	0.2	6.5
Fe	0.3	25
K	3	200
Li	0.003	<0.003
Mg	0.05	2.6
Mo	0.03	<0.03
Na	1	<1
Ni	0.3	<0.3
Rb	0.02	1.1
Zr	0.02	<0.02

Table 29 continued

Alum 4470		Ammonia-Alum 91480
Laboratory Reference		ASC/ 2666.005
	LOD	mg/kg
Al	3	58000
Ca	0.8	47
Cr	0.2	4.4
Fe	0.3	10
K	3	180
Li	0.003	<0.003
Mg	0.05	0.3
Mo	0.03	<0.03
Na	1	<1
Ni	0.3	<0.3
Rb	0.02	0.61
Zr	0.02	<0.02

Alum 4230		Ammonia-Alum AA0427
Laboratory Reference		2666.007
	LOD	mg/kg
Al	3	59000
Ca	2	<2
Cr	0.8	<0.8
Fe	0.8	32
K	5	5600
Li	0.02	0.2
Mg	0.5	42
Mo	0.02	0.08
Na	1	110
Ni	0.2	4.1
Rb	0.05	1.2
Zr	0.01	0.14

Table 30: Aluminium based vaccines for human use

Vaccin	Vaccine Details	Manufacturer / Supplier
Ambirix	Hepatitis A (inactivated) and hepatitis B (rDNA) (HAB) vaccine (adsorbed). Adsorbed on aluminium hydroxide, hydrated	GlaxoSmithKline
Cervarix	Human Papillomavirus vaccine [Types 16, 18] (Recombinant, adjuvanted, adsorbed) adsorbed on aluminium hydroxide, hydrated (Al(OH)3)	GlaxoSmithKline
Engerix-B	Hepatitis B (rDNA) vaccine, adsorbed (HBV) Adsorbed on aluminium hydroxide, hydrated	GlaxoSmithKline
Havrix	Havrix Junior Monodose Vaccine: Excipients - Aluminium hydroxide gel (3% w/w) Havrix Monodose Vaccine: Excipients - Aluminium hydroxide gel (3% w/w)	GlaxoSmithKline
Hepatyrix	Hepatitis A (inactivated) and Typhoid Polysaccharide vaccine (adsorbed). Adsorbed on aluminium hydroxide, hydrated	GlaxoSmithKline
Infanrix	Diphtheria and tetanus toxoids and pertussis antigens (PT, FHA, and pertactin) are individually adsorbed onto aluminum hydroxide.	GlaxoSmithKline
Infanrix-IPV	Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed) Adsorbed on aluminium hydroxide,hydrated	GlaxoSmithKline
inrix	Diphtheria and tetanus toxoids and pertussis antigens (inactivated PT, FHA, and pertactin) are individually adsorbed onto aluminum hydroxide.	GlaxoSmithKline
Pediarix	Diphtheria and tetanus toxoids and pertussis antigens (inactivated PT, FHA, and pertactin) are individually adsorbed onto aluminum hydroxide. The hepatitis B component is adsorbed onto aluminum phosphate.	GlaxoSmithKline
Twinrix	Twinrix Adult Vaccine: Hepatitis A (inactivated) and hepatitis B (rDNA) (HAB) vaccine (adsorbed). Adsorbed on aluminium hydroxide, hydrate Twinrix Paediatric Vaccine suspension: Hepatitis A (inactivated) and hepatitis B (rDNA) (HAB) vaccine (adsorbed). Adsorbed on aluminium hydroxide, hydrated	GlaxoSmithKline

Infanrix Hexa	Diptheria, tetanus, acellular pertussi, hepatitis B, inactivated poliomyelitis (DTPa-HBV-IPV), HiB adsorbed on Aluminium hydroxide, hydrated	GlaxoSmithKline
Infanrix HiB	Diphtheria (toxoid)/Tetanus (toxoid)/Pertussis antigens: PT/FHA/PN/ Haemophilus influenza type B (polysaccharide) adsorbed on Aluminium hydroxide	GlaxoSmithKline
Boostrix	Aluminum adjuvant (as aluminum hydroxide and aluminum phosphate), sodium chloride and water for injection.	GlaxoSmithKline
Boostrix-Polio	Diphtheria toxoid, tetanus toxoid, pertussis toxoid, poliovirus antigens Aluminum adjuvant (as aluminum salts), sodium chloride and water for injection.	GlaxoSmithKline
Fendrix	Hepatitis B (rDNA) vaccine (adjuvanted, adsorbed). Adsorbed on aluminium phosphate (0.5 milligrams Al3+ in total) Also contains alum as adjuvant	GlaxoSmithKline
Synflorix	Pneumococcal polysaccharide conjugate vaccine (adsorbed), adsorbed on aluminium phosphate	GlaxoSmithKline
Infanrix Penta	Diphtheria toxoid adsorbed on aluminium hydroxide ($Al(OH)_3$), tetanus toxoid, Bordetella pertussis antigens (pertussis toxoid, filamentous haemagglutinin, pertactin), hepatitis B surface antigen, poliovirus (inactivated) (type 1 (Mahoney strain), type 2 (MEF-1 strain), type 3 (Saukett strain))	GlaxoSmithKline
Daronrix	Whole virion, inactivated, containing antigen*: A/Vietnam/1194/2004 (H5N1) adjuvanted by Aluminium hydroxide Aluminium Phosphate	GlaxoSmithKline - Marketing Authorisation Holder Sächsisches Serumwerk Dresden (SSW) - Manufacturer
Infanrix-IPV+HIB	HIBERIX® reconstituted with INFANRIX™-IPV. Combined diphtheria, tetanus, acellular pertussis, inactivated poliomyelitis, Haemophilus influenzae type b vaccine. Aluminum adjuvant (as 0.5 mg aluminum salts).	GlaxoSmithKline

Avaxim ®	Excipients: 2-phenoxyethanol, formaldehyde, aluminum hydroxide (expressed as aluminum) AVAXIM® [Hepatitis A vaccine Inactivated] is a sterile, whitish, cloudy suspension. The active ingredient is a purified and formaldehyde-inactivated hepatitis A virus (HAV) obtained from the GBM strain, cultured on MRC-5 human diploid cells. HAV is adsorbed onto aluminum.	Sanofi
Avaxim ® - Pediatric	Aluminum hydroxide (expressed as aluminum)	Sanofi
ViVAXIM ®	Excipients: sodium chloride, disodium phosphate dihydrate, sodium dihydrogen phosphate dihydrate, aluminum hydroxide (expressed as aluminum), 2-phenoxyethanol, formaldehyde, polysorbate 80, Medium 199 Hanks This vaccine is a buffered solution (without phenol) of purified Salmonella typhi Vi capsular polysaccharide (Ty2 strain) and a suspension of purified and formaldehyde-inactivated HAV, obtained from GBM strain, cultured on MRC-5 human diploid cells. HAV is adsorbed onto aluminum.	Sanofi

Table 31: Aluminium based vaccines for veterinary use

Vaccine	Vaccine Details	Manufacturer / Supplier
CattleMaster 4+ VL5	CattleMaster® 4 is a freeze-dried preparation of chemically altered strains of IBR and PI3 virusses and modified live BRSV plus a liquid, adjuvanted preparation of inactivated cytopathic and noncytopathic BVD virus strains. The liquid component is used to rehydrate the freeze-dried component. Viral fractions are propagated on an established cell line. The product is adjuvanted with aluminium hydroxide to enhance the immune response.	Pfizer
Ingelvac® DART/AR4	Ingelvac® DART/AR4 are aluminium potassium sulphate based adjuvanted vaccines against progressive atrophic rhinitis (PAR), which consist of a 4-way bacterin-toxoid combination of Bordetella bronchiseptica and Pasteurella multocida type D. The vaccines provide cross protection against PAR caused by toxigenic Bb and Pm type A and have a very wide safety margin.	BOEHRINGER INGELHEIM
Resvac® 4/Somubac®	Somubac is prepared from selected strains of Haemophilus somnus which are grown serum-free in an environmentally controlled fermentation system and inactivated in such a manner as to maintain their immunogenic integrity. The bacterin is adjuvanted with aluminum hydroxide.	Pfizer
Pestorin Mormyx	Rabbit Hemorrhagic Disease, Killed type vaccine. Adjuvant - Aluminum hydroxide	Bioveta
Pestorin	Rabbit Hemorrhagic Disease Adjuvant - Aluminum hydroxide	Bioveta
MORIN	Rabbit Hemorrhagic Disease Type- Killed Adjuvant- Aluminum hydroxide	Dyntec
Hemoragivac	Rabbit Hemorrhagic Disease Adjuvant- Aluminum hydroxide	Institutul Pasteur
ARVILAP	Rabbit Hemorrhagic Disease Type- Killed Starin/Subtype- LO1 Adjuvant- Aluminum hydroxide	Laboratorios Ovejero
BTVPUR Alsap™ 1-8	Bluetongue Disease Type- Killed Starin/Subtype- Serotype 1, 8 Adjuvant- Aluminum hydroxide, saponin	Sanofi (Merial SAS (France))
BTVPUR Alsap™ 2-4	Bluetongue Disease Type- Killed Adjuvant- Aluminum hydroxide, saponin	Sanofi (Merial SAS (France))
BTVPUR Alsap™ 1	Bluetongue Disease Type- Killed Adjuvant- Aluminum hydroxide, saponin	Sanofi (Merial SAS (France))

BTVPUR Alsap™ 2	Bluetongue Disease Type- Killed Adjuvant- Aluminum hydroxide, saponin	Sanofi (Merial SAS (France))
BTVPUR Alsap™ 4	Bluetongue Disease Type- Killed Adjuvant- Aluminum hydroxide, saponin	Sanofi (Merial SAS (France))
BTVPUR Alsap™ 9	Bluetongue Disease Type- Killed Adjuvant- Aluminum hydroxide, saponin	Sanofi (Merial SAS (France))
BOVILIS® BTV8	Bluetongue Disease Type- Killed Adjuvant- Aluminum hydroxide, saponin	MSD Animal Health (Merck)
Clotisol®	This original-formula blood clotting suspension is used as an aid to stop bleeding in animals caused by minor cuts and wounds. Clotisol can be used effectively in nail trimmings, tail docking, ear cropping and on other minor bleeding sites. Ingredients: Ferric Sulfate, Aluminum Sulfate, Collagen Protein and Chlorxylenol in suspension.	Ceva Animal Health
Sal Bac®	Prevention of paratyphoid infection attributed to Salmonella typhimurium in pigeons. Sal Bac® is an inactivated bacterial vaccine adjuvanted with aluminum hydroxide. It also contains a patented biological adjuvant which acts as an immune enhancer to ensure the level of response to vaccination and provide long-lasting immunity	Ceva Animal Health
CEVAC® CORYMUNE 4K	CEVAC® CORYMUNE 4K contains an inactivated combination of Avibacterium paragallinarum serotypes A, B and C, and Salmonella Enteritidis strain, homogenized with aluminium hydroxide adjuvant and thiomersal as a preservative.	Ceva Animal Health
Hepatovax	Duck Virus Hepatitis Type- Live Starin/Subtype- E52 Adjuvant- Aluminum hydroxide	Sanofi (Merial SAS (France))
Septivac	Hemorrhagic Septicemia Type- Killed Adjuvant- Aluminum hydroxide	Institutul Pasteur
ITA-ND+IB+EDS	Egg Drop Syndrome Type- Killed Strain/ Subtype- B8/78	Laprovet
Lohmann Animal Health International	Newcastle Disease, Infectious Bronchitis, Egg Drop Syndrome Type-Killed Starin/Subtype- EDS 76	Lohmann Animal Health International

SCOURGUARD® 4KC	ScourGuard 4K is for the vaccination of healthy, pregnant cows and heifers as an aid in preventing diarrhea in their calves caused by bovine rotavirus (serotypes G6 and G10), bovine coronavirus, and enterotoxigenic strains of Escherichia colihaving the K99 pili adherence factor. ScourGuard 4K contains a liquid preparation of inactivated bovine rotavirus (serotypes G6 and G10) and coronavirus propagated on established cell lines and a K99 E. colibacterin. The vaccine is adjuvanted to enhance the immune response.	Pfizer
Salmonella Newport Bacterial Extract Vaccine with SRP® technology	The SRP antibodies generated by vaccination with Salmonella Newport Bacterial Extract vaccine have been shown to recognize the SRP's of several common Salmonella serotypes. Contains an emulsified adjuvant	Pfizer
ULTRABAC® 8	For use in healthy cattle and sheep as an aid in preventing blackleg caused by Clostridium chauvoei, malignant edema caused by Cl. septicum, bacillary hemoglobinuria caused by Cl. haemolyticum, black disease caused by Cl. novyi, gas gangrene caused by Cl. sordellii, and enterotoxemia and enteritis caused by Cl. perfringens Types B, C and D. Although Cl. perfringens Type B is not a significant problem in North America, immunity is provided by the beta toxoid of Type C and the epsilon toxoid of Type D. Ultrabac 8 consists of killed, standardized cultures of Cl. chauvoei, Cl. septicum, Cl. haemolyticum, Cl. novyi, Cl. sordellii, and Cl. perfringens Types C and D, with an adjuvant.	Pfizer
ULTRABAC® 7/SOMUBAC	For use in healthy cattle and calves three months of age or older as an aid in preventing blackleg caused by Clostridium chauvoei, malignant edema caused by Cl. septicum, black disease caused by Cl. novyi, gas-gangrene caused by Cl. sordellii, enterotoxemia and enteritis caused by Cl. perfringens Types B, C and D, and disease caused by Histophilus somni (Haemophilus somnus). Although Cl. perfringens Type B is not a significant problem in North America, immunity is provided by the beta toxoid of Type C and the epsilon toxoid of Type D. Ultrabac 7/Somubac consists of killed, standardized cultures of Cl. chauvoei, Cl. septicum, Cl. novyi, Cl. sordellii, Cl. perfringens Types C and D, and H. somni, with an adjuvant	Pfizer
Litterguard	The bacterin is prepared from chemically inactivated strains of E. coli. A sterile adjuvant is used to enhance the immune response.	Pfizer

DISTOX®-PLUS	This vaccine is made up of two components: a lyophilized (freeze-dried) vaccine containing a modified live distemper virus grown in tissue culture and combined with stabilizing agents; and a liquid fraction containing inactivated mink enteritis virus grown in a feline cell line, Clostridium botulinum Type C bacterin-toxoid, and inactivated Pseudomonas aeruginosa Serotypes 5, 6, 7-8, and 9 with an aluminum adjuvant	Merck Animal Health
COVEXIN® 8	Immunizing antigens of Cl. chauvoei, Cl. haemolyticum, Cl. novyi Type B, Cl. perfringens Types B, C & D, Cl. septicum and Cl. tetani, with potassium alum adjuvant	Merck Animal Health
MaxiVac Excell® 5.0	Inactivated vaccine containing three strains of swine influenza virus type A, subtype H1N1, and two strains of swine influenza virus type A, subtype H3N2, in a proprietary dual-acting adjuvant (Emunade®).	Merck Animal Health
M+PAC®	Mycoplasma Hyopneumoniae Bacterin, contains the proprietary dual adjuvant Emunade®	Merck Animal Health
M+RHUSIGEN®	Erysipelothrix Rhusiopathiae-Mycoplasma Hyopneumoniae Bacterin, contains the proprietary dual adjuvant Emunade®	Merck Animal Health
SCOURMUNE®	Escherichia Coli Bacterin pili types K88, K99, 987P and Type 1, Aluminum Hydroxide Adsorbed	Merck Animal Health
SCOURMUNE®-C	Clostridium Perfringens Type C - Escherichia Coli Bacterin - Toxoid, pili types K88, K99, 987P and Type 1, Aluminum Hydroxide Adsorbed	Merck Animal Health
TASVAX® 8	Antigens of Cl. chauvoei, Cl. haemolyticum, Cl. novyi Type B, Cl. perfringens Types B, C & D, Cl. septicum and Cl. tetani, with potassium alum adjuvant	Merck Animal Health
MaxiVac Excell®3	Swine Influenza Vaccine, H1N1 and H3N2, Killed Virus, Emunade® adjuvant	Merck Animal Health
Quantum Cat FeLV	Inactivated adjuvanted feline leukaemia viral antigens derived from subtypes A, B and C including feline oncornavirus–associated cell membrane antigen, Alhydrogel 2 % Al203 as adjuvant	Merck Animal Health
Tetanus Toxoid Concentrated	Tetanus Toxoid Concentrated is an inactivated vaccine containing ≥ 150 I.U. per dose (1 ml) purified tetanus toxoid. To enhance the immunogenicity the toxoid is adsorbed onto aluminium hydroxide	Merck Animal Health
Blackleg Vaccine	Suspension for injection containing five strains of Clostridium chauvoei formaldehyde inactivated cells/dose and equivalent toxoid inducing ≥ 0.5 guinea pig PD90 per strain. Also contains Aluminium hydroxide as an adjuvant and 0.26 mg/dose Thiomersal as preservative.	Merck Animal Health
Bovilis™ Bovipast RSP	An aqueous suspension for subcutaneous injection. One dose (5 ml) of the vaccine contains at least 105.5 TCID50 inactivated Bovine Respiratory Syncytial virus (strain EV 908) and at least 107.3 TCID50 Parainfluenza-3- virus (strain SF-4 Reisinger), together with 9 x 109 cells inactivated Mannheimia	Merck Animal Health

	(Pasteurella) haemolytica bacteria (serotype A1) propagated under conditions of iron restriction. Aluminium hydroxide and Quil A are included as adjuvants. Sodium timerfonate is included as a preservative.	
Bovilis BTV8	Active substance 1 ml dose contains: Bluetongue Virus Serotype 8 (prior to inactivation): 500 Antigenic Units *. (* inducing a virus neutralising antibody response in chickens of ≥5.0 log2). Adjuvants Aluminium hydroxide (as 100%) 16.7 mg and Saponin 0.31 mg	Merck Animal Health
Bovilis BVD	Bovilis BVD is an inactivated vaccine containing 50 ELISA units (EU) and inducing at least 4.6 log2 VN units per dose of cytopathogenic BVD virus strain C86. The virus is grown in cell culture, inactivated with beta-propiolactone, and adsorbed onto an aluminium salts adjuvant	Merck Animal Health
Bovivac S	Bovivac S is a suspension for injection containing inactivated cells of Salmonella dublin, strain S342/70 (1 x 109 cells/ml) and inactivated cells of Salmonella typhimurium, strain S341/70 (1 x 109 cells/ml). Bovivac S contains aluminium hydroxide as an adjuvant and thiomersal as a preservative.	Merck Animal Health
Bravoxin 10	Bravoxin 10 is a liquid vaccine for injection containing inactivated clostridial antigens with an aluminium potassium sulphate (alum) adjuvant	Merck Animal Health
Lambivac	Suspension for injection containing per ml Clostridium perfringens beta toxoid inducing ≥ 10 IU; Clostridium perfringens epsilon toxoid inducing ≥ 5 IU; and Clostridium tetani toxoid inducing ≥ 2.5 IU. Also contains Aluminium hydroxide as an adjuvant and 0.13 mg/ml Thiomersal as preservative.	Merck Animal Health
Leptavoid™ – H	Leptospira interrogans serovar hardjo 204 (inactivated), Adjuvant (10% alum)	Merck Animal Health
Rotavec Corona	Bovine rotavirus, strain UK-Compton, serotype G6 P5 (inactivated), Bovine coronavirus, strain Mebus (inactivated), E. coli F5 (K99) adhesin, also contains 1.5 ml Light Mineral Oil / emulsifier and 2.45 – 3.32 mg Aluminium hydroxide as adjuvants and 0.051 – 0.069 mg Thiomersal as preservative.	Merck Animal Health

Heptavac-P Plus	Combined 7 in 1 Clostridial plus Pasteurella vaccine. An opaque fluid vaccine containing per ml: Clostridium perfringens beta toxoid : inducing ≥ 10 IU ; Clostridium perfringens epsilon toxoid : inducing ≥ 5 IU ; Clostridium septicum: toxoid : inducing ≥ 2.5 IU; Clostridium tetani toxoid: inducing ≥ 2.5 IU; Clostridium novyi toxoid :inducing ≥ 3.5 IU; Clostridium chauvoei cells and equivalent toxoid : inducing ≥ 0.5 guinea pig PD90, formalin killed cells of the epidemiologically most important serotypes of Mannheimia haemolytica and Pasteurella trehalosi grown under iron restricted conditions: 5x108 cells per strain in buffered physiological saline adsorbed onto aluminium hydroxide.	Merck Animal Health
Ovipast Plus	Per 1 ml: 5 x 108 formalin killed cells each of M. haemolytica strains A1 (S1006/77), A2 (S1126/92), A6 (S1084/81), A7 (S1078/81), A9 (S994/77) and P. trehalosi strains T3 (S1109/84), T4 (S1085/81), T10 (S1075/81), T15 (S1105/84) Alhydrogel, Thiomersal.	Merck Animal Health
Ovivac-P Plus	Cl. perfringens type D, strain 603 epsilon toxoid, Cl. septicum, strain S1110/85 toxoid, Cl. tetani, strain 51123/91 toxoid, Cl. chauvoei, strains 655, 656, 657, 658, 1048 cells and equivalent toxoid, M. haemolytica, strains A1, A2, A6, A7, A9, P.trehalosi, strains T3, T4, T10, T15 Adjuvant: Aluminium hydroxide	Merck Animal Health
Porcilis Coli 6C	Porcilis Coli 6C is a liquid vaccine with a precipitate which resuspends on shaking. Each 5 ml dose contains cell-free pilus antigens of E. coli, strains K88ab (100–135 units); K88ac (100–135 units); K99 (190–250 units); 987P (2900–3100 units) and purified toxoid of C. perfringens Types B, C and D (together contributing not less than 300 International Unit equivalents of beta toxoid and not less than 200 International Unit equivalents of epsilon toxoid) all absorbed onto aluminium hydroxide (≤ 15mg AL3+)	Merck Animal Health
Nobilis Salenvac T	Inactivated cells of Salmonella Enteritidis, strain PT 4, Inactivated cells of Salmonella Typhimurium, strain DT104, Adjuvant: aluminium hydroxide	Merck Animal Health
Parvo Shield L5E	Parvo Shield L5E provides solid protection against parvovirus, five strains of leptospirosis and erysipelas, aluminium hydroxide as adjuvant	Novartis Animal Health
Prefarrow Shield 9	For use in healthy pregnant swine as an aid in the control of disease in piglets caused by Bordetella bronchiseptica, Clostridium perfringens Type C, Erysipelothrix rhusiopathiae, K88, K99, 987P, and F41 piliated Escherichia coli, and the toxin of Pasteurella multocida Types A and D. Adjuvant: Aluminum hydroxide.	Novartis Animal Health
Rhini Shield™ TX4	Rhini Shield TX4 helps protect pigs against atrophic rhinitis caused by Bordetella bronchiseptica or the toxin of Pasteurella multocida Types A and D, erysipelas, and pneumonia caused by P. multocida Type A. Adjuvant: Aluminium hydroxide	Novartis Animal Health

Canigen Rabies	Inactivated vaccine containing > 2 I.U. Rabies virus strain Pasteur RIV per dose. Also contains Aluminium phosphate as an adjuvant.	Virbac
Leucofeligen FeLV/RCP	Live Feline Calicivirus (strain F9), Live Feline Viral Rhinotracheitis virus (strain F2), Live Feline Panleucopaenia virus (strain LR 72), Minimum quantity of purified p45 FeLV-envelope antigen 102 microgrammes, Adjuvants: 3% aluminium hydroxide gel	Virbac
Leucogen	The vaccine contains the purified p45 FeLV-envelope antigen, obtained by genetic recombination of the E. Coli strain. The antigenic suspension is adjuvanted with an aluminium hydroxide gel	Virbac
COLIVAC	INACTIVATED, ADJUVANTATED VACCINE AGAINST ENTEROTOXIGENIC COLIBACILLARY DIARRHEA IN PIGLETS, Adjuvant: Aluminum hydroxide	Insitut Pastuer Romania
EMFIVAC	INACTIVATED VACCINE AGAINST BLACKLEG, Adjuvant: Aluminum hydroxide	Insitut Pastuer Romania
LEPTOVAC	INACTIVATED VACCINE AGAINST *LEPTOSPIRA SPP.* INFECTIONS IN CATTLE, HORSES, SHEEP AND SWINE, Adjuvant: Aluminum hydroxide	Insitut Pastuer Romania
MAMIVAC	INACTIVATED VACCINE AGAINST GANGRENOUS MASTITIS IN SHEEP AND GOATS, Adjuvant: Aluminum hydroxide	Insitut Pastuer Romania
ROMPERVAC ABCD	INACTIVATED VACCINE AGAINST *CL. PERFRINGENS* ENTEROTOXIEMIA IN CATTLE AND SHEEP, Adjuvant: Aluminum hydroxide	Insitut Pastuer Romania
SALMOVIN	VACCINE AGAINST *SALMONELLA* ABORTION IN SHEEP, Adjuvant: Aluminum hydroxide	Insitut Pastuer Romania
ARVILAP	Active immunisation against Haemorrhagic Viral Disease of rabbits, adsorbed on aluminium hydroxide	Ovejero Laboratorios
DOG-VAC R	Active inmunization against Rabies in dogs, cats and cattle, adsorbed on aluminium hydroxide	Ovejero Laboratorios
INMUBOV INACTIVADO	Virus Rinotraqueitis/Vulvovaginitis (IBR/IPV), Virus Parainfluenza-3 (PI-3), Virus Diarrea Vírica Bovina (BVD). Adsorbed on aluminium hydroxide.	Ovejero Laboratorios
NEO-VAKY MR	Erysipelothrix rhusiopathiae, Adsorbed on aluminium hydroxide.	Ovejero Laboratorios
COVEXIN® PLUS	Clostridium Chauvoei-Septicum-Haemolyticum-Novyi-Sordellii-Tetani-Perfringens Types C & D Bacterin-Toxoid	Merck Animal Health
NOBIVAC® Canine Flu H3N8	Nobivac® Canine Flu H3N8 is recommended for the vaccination of healthy dogs at 6 weeks of age or older as an aid in the control of disease associated with canine influenza virus infection. The type A, subtype H3N8 virus has been chemically inactivated and combined with an adjuvant designed to enhance the immune response.	Merck Animal Health

NOBIVAC® Feline 1-HCP + FeLV	Nobivac® Feline 1-HCP + FeLV vaccine is a combination vaccine that unites the benefits of Nobivac® Feline 1-HCP and Nobivac® FeLV in one vaccination. Nobivac® Feline 1-HCP vaccine is a modified live virus vaccine for the vaccination of healthy cats as an aid in the prevention of disease caused by feline rhinotracheitis, calici, and panleukopenia viruses. Nobivac® FeLV vaccine is recommended for the vaccination of healthy cats as an aid in the prevention of lymphoid tumors caused by, and diseases associated with, feline leukemia virus (FeLV) infection. The virus has been chemically inactivated and combined with a proprietary adjuvant designed to enhance the immune response.	Merck Animal Health
CAV-VAC®	CAV-VAC is a live virus vaccine, prepared from a modified U.S. field isolate. The vaccine is produced using specific pathogen free (SPF) substrates, and contains live chicken anemia vaccine virus suspended in wing web diluent.	Merck Animal Health
CONTINUUM™ DAP-R 3-YEAR DOI	CONTINUUM™ DAP-R is a modified live virus vaccine containing attenuated strains of canine distemper virus (CDV), adenovirus type-2 (CAV-2), parvovirus (CPV), and inactivated rabies virus. CONTINUUM™ DAP-R is presented in a desiccated form with rabies diluent provided for reconstitution.	Merck Animal Health
CONTINUUM™ P 3-YEAR DOI	CONTINUUM™ P is a modified live virus vaccine containing an attenuated strain of canine parvovirus (CPV). CONTINUUM™ P is presented in a desiccated form with sterile diluent provided for reconstitution.	Merck Animal Health
FLU AVERT® I.N. VACCINE	This is a lyophilized preparation containing an attenuated, cold adapted, viable A equine-2 influenza virus. Contains no preservatives.	Merck Animal Health
FVR® C	FVR® C vaccine is a modified live virus vaccine for the vaccination of healthy cats 8 to 9 weeks of age or older as an aid in the prevention of disease caused by feline rhinotracheitis and calici viruses.	Merck Animal Health
FVR® C-P	VR® C-P vaccine is a combination of antigens for convenient use in healthy cats 8 to 9 weeks of age or older as an aid in the prevention of disease caused by feline rhinotracheitis, feline calici and feline panleukopenia viruses. The feline rhinotracheitis-calici component is a lyophilized suspension of modified live viruses propagated in a stable cell line of feline origin and backfilled with an inert gas. The feline panleukopenia virus is propagated in a stable cell line of feline origin. The virus has been chemically inactivated and determined to be non-viricidal when used as a diluent to rehydrate the feline rhinotracheitis-calici vaccine.	Merck Animal Health
Galaxy™ D	Galaxy™ D vaccine is a modified live virus vaccine for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine distemper.	Merck Animal Health

Galaxy™ Pv	Galaxy™ Pv vaccine is a modified live virus vaccine containing a CPV Type 2b strain for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine parvovirus.	Merck Animal Health
Intra-Trac®-II ADT	Canine Parainfluenza-Bordetella Bronchiseptica Vaccine (Modified Live Virus, Avirulent Live Culture)	Merck Animal Health
JENCINE® ERC	Bovine Rotavirus-Coronavirus Vaccine, Killed Virus, Clostridium Perfringens Types C & D-Escherichia Coli Bacterin-Toxoid	Merck Animal Health
LT-IVAX®	Fowl Laryngotracheitis Vaccine	Merck Animal Health
NOBIVAC® Canine 1-DAPPv	Nobivac® Canine 1-DAPPv vaccine is a modified live virus vaccine for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine distemper virus, adenovirus type 1 (hepatitis) and adenovirus type 2 (respiratory disease), canine parainfluenza virus, and canine parvovirus	Merck Animal Health
NOBIVAC® Canine 1-DAPPv +Cv	Nobivac® Canine 1-DAPPv+Cv vaccine is a combination vaccine that unites the benefits of Nobivac® Canine 1-DAPPv and Nobivac® Canine 1-Cv in one vaccination. Nobivac® Canine 1-DAPPv vaccine is a modified live virus vaccine for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine distemper virus, adenovirus type 1 (hepatitis), adenovirus type 2 (respiratory disease), canine parainfluenza virus, and canine parvovirus. Nobivac® Canine 1-Cv vaccine (feline enteric coronavirus) is a killed virus vaccine for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine coronavirus infection. The coronavirus has been chemically inactivated and combined with an adjuvant designed to enhance the immune response.	Merck Animal Health
NOBIVAC® Canine 1-DAPPvL2	Nobivac® Canine 1-DAPPvL2 vaccine is a modified live virus vaccine which is combined with an inactivated Leptospira canicola and Leptospira icterohaemorrhagiae bacterin for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine distemper virus, canine adenovirus type 1 (hepatitis), canine adenovirus type 2 (respiratory disease), canine parainfluenza virus, and canine parvovirus and against Leptospiral disease due to L. canicola or L. icterohaemorrhagiae	Merck Animal Health
NOBIVAC® Canine 1-DAPPvL2+Cv	Nobivac® Canine 1-DAPPvL2+Cv vaccine is a combination vaccine that unites the benefits of Nobivac® Canine 1-DAPPvL2 and Nobivac® Canine 1-Cv in one vaccination. Nobivac® Canine 1-DAPPvL2 vaccine is a modified live virus vaccine which is combined with an inactivated Leptospira canicola and Leptospira icterohaemorrhagiae bacterin for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine distemper virus, canine adenovirus type 1 (hepatitis), canine adenovirus type 2 (respiratory disease), canine parainfluenza virus, and canine parvovirus and against Leptospiral disease due to L. canicola or L. icterohaemorrhagiae. Nobivac® Canine 1-Cv vaccine	Merck Animal Health

	(feline enteric coronavirus) is a killed virus vaccine for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine coronavirus infection. The coronavirus has been chemically inactivated and combined with an adjuvant designed to enhance the immune response.	
Nobivac® Canine 1-DAPPv+L4	Nobivac® Canine 1-DAPPv+L4 vaccine is a combination vaccine that unites the benefits of Nobivac® Canine 1-DAPPv and Nobivac® Lepto4 in one vaccination. Nobivac® Canine 1-DAPPv vaccine is a modified live virus vaccine for the vaccination of healthy dogs as an aid in the prevention of disease caused by canine distemper virus, adenovirus type 1 (hepatitis), canine parainfluenza virus, and canine parvovirus. Nobivac® Lepto4 bacterin is recommended for the vaccination of healthy dogs as an aid in the prevention of disease and mortality caused by L. canicola, L. icterohaemorrhagiae, L. pomona, or L. grippotyphosa.	Merck Animal Health
NOBIVAC® Canine 3-DA	Nobivac® Canine 3-DA is a modified live virus vaccine containing attenuated strains of canine distemper virus (CDV) and adenovirus type 2 (CAV-2)	Merck Animal Health
NOBIVAC® Canine 3-DAPv	Nobivac® Canine 3-DAPv is a modified live virus vaccine containing attenuated strains of canine distemper virus (CDV), adenovirus type 2 (CAV-2), and parvovirus (CPV)	Merck Animal Health
NOBIVAC® Canine 3-Pv	Nobivac® Canine 3-Pv is a modified live virus vaccine containing an attenuated strain of canine parvovirus (CPV)	Merck Animal Health
NOBIVAC® Feline 1-HCP	Nobivac® Feline 1-HCP vaccine is a modified live virus vaccine	Merck Animal Health
NOBIVAC® Feline 1-HCP + FeLV	Nobivac® Feline 1-HCP + FeLV vaccine is a combination vaccine that unites the benefits of Nobivac® Feline 1-HCP and Nobivac® FeLV in one vaccination. Nobivac® Feline 1-HCP vaccine is a modified live virus vaccine for the vaccination of healthy cats as an aid in the prevention of disease caused by feline rhinotracheitis, calici, and panleukopenia viruses. Nobivac® FeLV vaccine is recommended for the vaccination of healthy cats as an aid in the prevention of lymphoid tumors caused by, and diseases associated with, feline leukemia virus (FeLV) infection. The virus has been chemically inactivated and combined with a proprietary adjuvant designed to enhance the immune response.	Merck Animal Health
NOBIVAC® Feline 1-HCPCh	Nobivac® Feline 1-HCPCh vaccine is a modified live virus and *chlamydia* vaccine for the vaccination of healthy cats as an aid in the prevention of disease caused by feline rhinotracheitis, calici, and panleukopenia viruses and *Chlamydia psittaci*.	Merck Animal Health

NOBIVAC® Feline 1-HCPCh + FeLV	Nobivac® Feline 1-HCPCh + FeLV vaccine is a combination vaccine that unites the benefits of Nobivac® Feline 1-HCPCh and Nobivac® FeLV in one vaccination. Nobivac® Feline 1-HCPCh vaccine is a modified live virus and chlamydia vaccine for the vaccination of healthy cats as an aid in the prevention of disease caused by feline rhinotracheitis, calici, and panleukopenia viruses and Chlamydia psittaci. Nobivac® FeLV vaccine is recommended for the vaccination of healthy cats as an aid in the prevention of lymphoid tumors caused by, and diseases associated with, feline leukemia virus (FeLV) infection. The virus has been chemically inactivated and combined with a proprietary adjuvant designed to enhance the immune response.	Merck Animal Health
NOBIVAC® Feline 3-HC	Nobivac® Feline 3-HC is a modified live virus vaccine containing attenuated strains of feline rhinotracheitis virus (FRV) and calicivirus (FCV)	Merck Animal Health
NOBIVAC® Feline 3-HCP	Nobivac® Feline 3-HCP is a modified live virus vaccine containing attenuated strains of feline rhinotracheitis virus (FRV), calicivirus (FCV), and panleukopenia virus (FPV)	Merck Animal Health
NOBIVAC® Feline 3-P	Nobivac® Feline 3-P is a modified live virus vaccine containing an attenuated strain of feline panleukopenia virus (FPV).	Merck Animal Health
Nobivac® FeLV	Nobivac® FeLV vaccine is recommended for the vaccination of healthy cats as an aid in the prevention of lymphoid tumors caused by, and diseases associated with, feline leukemia virus (FeLV) infection. The virus has been chemically inactivated and combined with a proprietary adjuvant designed to enhance the immune response.	Merck Animal Health
NOBIVAC® Intra-Trac®3 ADT	Nobivac® Intra-Trac®3 ADT† vaccine is for use as an aid in the prevention of disease associated with canine adenovirus type 2, canine parainfluenza virus and Bordetella bronchiseptica infection in healthy dogs 3 weeks of age or older	Merck Animal Health
NOBIVAC® Intra-Trac® KC	Nobivac® Intra-Trac® KC is a modified live intranasal vaccine containing attenuated canine parainfluenza virus and Bordetella bronchiseptica avirulent live culture for the vaccination of healthy susceptible puppies and dogs for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine parainfluenza and B. bronchiseptica	Merck Animal Health
NOBIVAC® Lepto4	Nobivac® Lepto4 bacterin is recommended for the vaccination of healthy dogs as an aid in the prevention of disease and mortality caused by L. canicola, L. icterohaemorrhagiae, L. pomona, and L. grippotyphosa; the prevention of leptospiruria (shedding of Leptospira in the urine) caused by L. canicola, L. icterohaemorrhagiae, and L. grippotyphosa; and as an aid in the prevention of leptospiruria caused by L. pomona.	Merck Animal Health
NOBIVAC® LYME	Nobivac® Lyme bacterin contains two inactivated isolates of Borrelia burgdorferi	Merck Animal Health

NOBIVAC® Puppy-DPv	Nobivac® Puppy-DPv is a modified live virus vaccine containing attenuated strains of canine distemper virus (CDV) and parvovirus (CPV)	Merck Animal Health
PARAPAC®	Contains chemically-inactivated and adjuvanted cultures of Haemophilus parasuis.	Merck Animal Health
PROGARD®-5	PROGARD®-5 is a modified live virus vaccine containing attenuated strains of canine distemper virus, adenovirus type 2, parainfluenza virus and parvovirus grown in the PRO-CELL STABLE CELL LINE™	Merck Animal Health
PROGARD®-6	PROGARD®-6 is composed of a lyophilized, modified live virus vaccine (Progard®-5) containing attenuated strains of canine distemper virus, adenovirus type 2, parainfluenza virus and parvovirus grown in the Pro-Cell Stable Cell Line™, and of a component with inactivated canine coronavirus vaccine as the diluent.	Merck Animal Health
PROSYSTEM® RCE	The Porcine Rotavirus vaccine contains two modified live G serotypes 5 and 4 of Serogroup A rotavirus, in desiccated form. The C. perfringens-E. coli bacterin-toxoid is a purified, adjuvanted liquid product containing the C. perfringens type C toxoid and four major E. coli pilus antigens - K88, K99, F41 and 987P. Each serial of ProSystem® CE (C. perfringens - E. coli bacterin-toxoid) is demonstrated to be compatible (non-viricidal) with ProSystem® Rota (Porcine Rotavirus vaccine) and therefore can be used as a diluent when packaged with the viral vaccine.	Merck Animal Health
RHINOGEN® BPE	A chemically inactivated, adjuvanted culture of B. bronchiseptica, E. rhusiopathiae and P. multocida nontoxigenic type A and toxigenic type D.	Merck Animal Health
SOW BAC® CE II	A chemically inactivated, adjuvanted product containing B. bronchiseptica, C. perfringens type C toxoid, E. rhusiopathiae, four major E. coli antigens (K88, K99, F41, and 987P) and P. multocida nontoxigenic type A and toxigenic type D.	Merck Animal Health
VISTA® 5 L5 SQ	The reconstituted vaccine-bacterin product contains modified-live cultures of bovine rhinotracheitis virus (IBRV), bovine virus diarrhea virus (BVD) (Types 1 & 2), parainfluenza3 virus (PI3), and bovine respiratory syncytial virus (BRSV) and inactivated cultures of Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae, and L. pomona with a proprietary adjuvant.	Merck Animal Health
VISTA® 5 SQ	The reconstituted vaccine product contains modified-live cultures of bovine rhinotracheitis virus (IBRV), bovine virus diarrhea virus (BVDV) (Types 1 & 2); parainfluenza3 (PI3) virus and bovine respiratory syncytial virus (BRSV).	Merck Animal Health
VISTA® 5 VL5 SQ	The reconstituted vaccine-bacterin product contains modified-live cultures of bovine rhinotracheitis virus (IBRV), bovine virus diarrhea virus (BVD) (Types 1 & 2), parainfluenza3 virus (PI3), and bovine respiratory syncytial virus (BRSV) and inactivated cultures of Campylobacter fetus and Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae, and L. pomona with a proprietary adjuvant	Merck Animal Health

L5 SQ	The bacterin product contains inactivated cultures of Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae, and L. pomona with a proprietary adjuvant.	Merck Animal Health
PROSYSTEM® CE	A purified, adjuvanted product containing four major E. coli antigens (K88, K99, F41 and 987P) and C. perfringens type C toxoid.	Merck Animal Health
PROSYSTEM® RCE	The Porcine Rotavirus vaccine contains two modified live G serotypes 5 and 4 of Serogroup A rotavirus, in desiccated form. The C. perfringens-E. coli bacterin-toxoid is a purified, adjuvanted liquid product containing the C. perfringens type C toxoid and four major E. coli pilus antigens - K88, K99, F41 and 987P.	Merck Animal Health
PROSYSTEM® TREC	The bacterin-toxoid diluent (ProSystem® CE) is a purified, adjuvanted liquid product containing four major E coli pilus antigens - K88, K99, F41 and 987P, and C. perfringens type C toxoid	Merck Animal Health
Clostri Shield® 7	Clostri Shield 7 is proven effective against disease-causing clostridial bacteria Proprietary dual-component adjuvant system for optimum immune response Highly syringeable/easily resuspended	Novartis Animal Health
Clostridium Perfringens Type A Toxoid	Clostridium Perfringens Type A Toxoid, Contains a proprietary dual-component adjuvant system	Novartis Animal Health

The following terms used in this specification are acknowledged as Registered Trade Marks:

"Triton", "Falcon", "UltiMate", "Infors", "Multitron", "LoBind", "Kleenpak", "Eppendorf", "Superose", "Millipore", "Steriflip", "BioTek" and "Tween".

Preferred aspects

Aspect 1. A method for preparing an aqueous composition comprising aluminium and a protein said method comprising

- combining an aluminium-salt, said protein and water to produce said aqueous composition and

- determining the level of a heavy metal in the aqueous composition and/or the aluminium-salt.

Aspect 2. A method for preparing an aqueous composition comprising aluminium and a protein said method comprising

- preparing or selecting an aluminium-salt that is able to provide an aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition and

- combining said aluminium salt, said protein and water to produce said aqueous composition.

Aspect 3. A method according to aspect 1-2, further comprising buffering said aqueous composition at a pH of between 6.5 and 8.5, preferably at a pH between 7.5 and 8.5.

Aspect 4. A method according to aspect 1-3, further comprising packaging aliquots of said aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition in separate air-tight storage containers.

Aspect 5. A method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions and precipitating

said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate.

Aspect 6. A method according to aspect 5, wherein the precipitate is selected that is able to provide an aqueous composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition.

Aspect 7. An aqueous composition comprising a protein and an aluminium-salt, said composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition.

Aspect 8. An aqueous composition according to aspect 7, which has been stored at temperatures higher than 20°C for at least 1 month.

Aspect 9. An aqueous composition according to aspect 7 having a shelf-life of at least 20 month.

Aspect 10. An aqueous composition according to aspect 7-9, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr, Pb, Rb and Mo.

Aspect 11. An aqueous composition according to aspect 7-10, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V.

Aspect 12. An aqueous composition according to aspect 7-11, wherein said heavy metal is selected from Cu or Ni.

Aspect 13. An aqueous composition according to aspect 7-12, wherein said heavy metal is present in ionic form.

Aspect 14. An aqueous composition according to aspect 7-13, wherein the aluminium-salt is aluminiumhydroxide (Al(OH)3) or aluminiumphosphate (AlPO4).

Aspect 15. An aqueous composition according to aspect 7-14, further comprising a reactive compound.

Aspect 16. An aqueous composition according to aspect 15, wherein the reactive compound is selected from the group consisting of a redox active compound, a radical building compound, a stabilizing compound and a combination of any thereof.

Aspect 17. An aqueous composition according to aspect 15-16, wherein the reactive compound is selected from the group consisting of formaldehyde, ethanol, chlorophorm, trichloroethylene, acetone, triton-X-100, deoxycholate, diethylpyrocarbonate, sulphite, $Na_2S_2O_5$, beta-proprio-lacton, polysorbate such as Tween 20®, Tween 80®, O_2, phenol, pluronic type copolymers, and a combination of any thereof.

Aspect 18. An aqueous composition according to aspect 7-17, comprising between 5 µg/ml and 50 mg/ml aluminium.

Aspect 19. An aqueous composition according to aspect 7-18, comprising between 50 µg/ml and 5 mg/ml aluminium.

Aspect 20. An aqueous composition according to aspect 7-19, comprising between 5 ppb and 250 ppb Fe based on the weight of the aqueous composition.

Aspect 21. An aqueous composition according to aspect 7-20, comprising less than 3 ppb Cu based on the weight of the aqueous composition.

Aspect 22. An aqueous composition according to aspect 7-21, comprising less than 40 ppb Ni based on the weight of the aqueous composition.

Aspect 23. An aqueous composition according to aspect 7-22, wherein said protein is a therapeutic and/or a vaccine.

Aspect 24. An aqueous composition according to aspect 7-23, wherein said protein is a viral or bacterial protein.

Aspect 25. An aqueous composition according to aspect 7-24, wherein said viral protein is a protein of the Japanese encephalitis virus or a protein of the *Pseudomonas aeruginosa* bacterium.

Aspect 26. An aqueous composition according to aspect 7-25, wherein said protein is protein within a formaldehyde inactivated virus particles.

Aspect 27. An aqueous composition according to aspect 7-26, further comprising sulphite.

Aspect 28. An aqueous composition according to aspect 7-27, obtained by a method according to aspect 1-6.

Aspect 29. A vaccine comprising an aqueous composition according to aspect 7-28.

Aspect 30. An aluminium hydroxide concentrate that a) comprises 10 mg/ml of said aluminium hydroxide and b) less than 7 microgram heavy metal, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 31. An aluminium hydroxide concentrate that comprises 10 mg/ml aluminium hydroxide and less than 7 microgram heavy metal, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 32. An aluminium salt concentrate that comprises less than 7ppm heavy metal based on the weight of the concentrate, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 33. An aluminium salt concentrate that comprises less than 700ppm heavy metal based on the weight of the aluminium salt, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 34. An aluminium hydroxide concentrate that comprises less than 700ppm heavy metal based on the weight of the aluminium hydroxide, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 35. A method for preparing an aqueous composition comprising aluminium, a reactive compound and a protein said method comprising

- preparing or selecting an aluminium-salt that is able to provide an aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition and
- combining said aluminium salt, said protein, reactive compound and water to produce said aqueous composition.

Aspect 36. The method for preparing an aqueous composition according to aspect 35, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr, Pb, Rb and Mo.

Aspect 37. The method for preparing an aqueous composition according to aspect 35-36, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V.

Aspect 38. The method for preparing an aqueous composition according to aspect 35-37, wherein said heavy metal is selected from Cu or Ni.

Aspect 39. The method for preparing an aqueous composition according to aspect 35-38, wherein said heavy metal is present in ionic form.

Aspect 40. The method for preparing an aqueous composition according to aspect 35-39, wherein the aluminium-salt is aluminiumhydroxide (Al(OH)3) or aluminiumphosphate (AlPO4).

Aspect 41. The method for preparing an aqueous composition according to aspect 35-40, wherein the reactive compound is selected from the group consisting of a redox active compound, a radical building compound, a stabilizing compound and a combination of any thereof.

Aspect 42. The method for preparing an aqueous composition according to aspect 35-41, wherein the reactive compound is selected from the group consisting of formaldehyde, ethanol, chlorophorm, trichloroethylene, acetone, triton-X-100, deoxycholate, diethylpyrocarbonate, sulphite, $Na_2S_2O_5$, beta-proprio-lacton, polysorbate such as Tween 20®, Tween 80®, O_2, phenol, pluronic type copolymers, and a combination of any thereof.

Aspect 43. The method for preparing an aqueous composition according to aspect 35-42, comprising between 5 µg/ml and 50 mg/ml aluminium.

Aspect 44. The method for preparing an aqueous composition according to aspect 35-43, comprising between 50 µg/ml and 5 mg/ml aluminium.

Aspect 45. The method for preparing an aqueous composition according to aspect 35-44, comprising between 5 ppb and 250 ppb Fe based on the weight of the aqueous composition.

Aspect 46. The method for preparing an aqueous composition according to aspect 35-45, comprising less than 3 ppb Cu based on the weight of the aqueous composition.

Aspect 47. The method for preparing an aqueous composition according to aspect 35-46, comprising less than 40 ppb Ni based on the weight of the aqueous composition.

Aspect 48. A method for preparing an aqueous composition according to aspect 35-47, further comprising buffering said aqueous composition at a pH of between 6.5 and 8.5, preferably 7.5 and 8.5.

Aspect 49. A method for preparing an aqueous composition according to aspect 35-48, further comprising packaging aliquots of said aqueous composition in separate air-tight storage containers.

Aspect 50. A method for prevention and/or treatment of a subject in need thereof that comprises the administration of an aqueous composition comprising an effective dose of an antigen, an aluminium compound, a reactive compound and less than 350ppb heavy metal based on the weight of the aqueous composition.

Aspect 51. A method for prevention and/or treatment of a subject in need thereof that comprises the administration of an effective dose of a composition according to aspect 7-29.

An aluminium hydroxide concentrate that a) comprises 10 mg/ml of said aluminium hydroxide and b) less than 7 microgram heavy metal, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 52. An aluminium hydroxide concentrate that comprises 10 less than 1.4 microgram heavy metal, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 53. An aluminium salt concentrate that comprises less than 1.4ppm heavy metal based on the weight of the concentrate, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 54. An aluminium salt concentrate that comprises less than 140ppm heavy metal based on the weight of the aluminium salt, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Aspect 55. An aluminium hydroxide concentrate that comprises less than 140ppm heavy metal based on the weight of the aluminium hydroxide, for use in the manufacture of a medicine, preferably for use in the manufacture of a vaccine.

Claims

1. A method for preparing an aqueous composition comprising aluminium, a reactive compound and a protein said method comprising

- preparing or selecting an aluminium-salt that is able to provide an aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition and

- combining said aluminium salt, said reactive compound, said protein and water to produce said aqueous composition.

2. A method according to claim 1, further comprising buffering said aqueous composition at a pH of between 6.5 and 8.5.

3. A method according to claim 1-2, further comprising packaging aliquots of said aqueous composition having less than 350 ppb heavy metal based on the weight of the aqueous composition in separate air-tight storage containers.

4. A method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, wherein the precipitate is selected that is able to provide an aqueous composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition.

5. An aqueous composition comprising a protein and an aluminium-salt, said composition comprising less than 350 ppb heavy metal based on the weight of the aqueous composition.

6. An aqueous composition according to claim 5, which has been stored at temperatures higher than 20°C for at least 1 month.

7. An aqueous composition according to claim 5-6, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr, Pb, Rb and Mo.

8. An aqueous composition according to claim 5-7, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V.

9. An aqueous composition according to claim 5-8, wherein said heavy metal is selected from Cu or Ni.

10. An aqueous composition according to claim 5-9, wherein said heavy metal is present in ionic form.

11. An aqueous composition according to claim 5-10, wherein the aluminium-salt is aluminiumhydroxide (Al(OH)3) or aluminiumphosphate (AlPO4).

12. An aqueous composition according to claim 5-11, wherein the aluminium-salt is aluminiumhydroxide (Al(OH)3).

13. An aqueous composition according to claim 5-12, wherein the reactive compound is selected from the group consisting of a redox active

compound, a radical building compound, a stabilizing compound and a combination of any thereof.

14. An aqueous composition according to claim 5-13, wherein the reactive compound is selected from the group consisting of formaldehyde, ethanol, chlorophorm, trichloroethylene, acetone, triton-X-100, deoxycholate, diethylpyrocarbonate, sulphite, $Na_2S_2O_5$, beta-proprio-lacton, polysorbate such as Tween 20®, Tween 80®, O_2, phenol, pluronic type copolymers, and a combination of any thereof.

15. An aqueous composition according to claim 5-14, comprising between 5 µg/ml and 50 mg/ml aluminium.

16. An aqueous composition according to claim 5-15, comprising between 50 µg/ml and 5 mg/ml aluminium.

17. An aqueous composition according to claim 5-16, comprising between 5 ppb and 250 ppb Fe based on the weight of the aqueous composition.

18. An aqueous composition according to claim 5-17, comprising less than 3 ppb Cu based on the weight of the aqueous composition.

19. An aqueous composition according to claim 5-18, comprising less than 40 ppb Ni based on the weight of the aqueous composition.

20. An aqueous composition according to claim 5-19, wherein said protein is a therapeutic and/or a vaccine.

21. An aqueous composition according to claim 5-20, wherein said protein is a viral or bacterial protein.

22. An aqueous composition according to claim 5-21, wherein said viral protein is a protein of the Japanese encephalitis virus or a protein of the *Pseudomonas aeruginosa* bacterium.

23. An aqueous composition according to claim 5-22, wherein said protein is protein within a formaldehyde inactivated virus particles.

24. An aqueous composition according to claim 5-23, further comprising sulphite.

25. An aqueous composition according to claim 5-24, obtained by a method according to claim 1.

26. A vaccine comprising an aqueous composition according to claim 5-24.

27. A method, aqueous composition or vaccine according to any one of claims 1-26, wherein the heavy metal content is less than 450 ppm based on the weight of the aluminium (gram/gram) in the aqueous composition.

28. A method according to any one of claims 1-27, wherein the Fe content of the aqueous composition is less than 700 ppm based on the weight of the aluminium in the composition; the Ni content is less than 18 ppm based on the weight of the aluminium in the composition; or the Cu content is less than 2,5 ppm based on the weight of the aluminium in the aqueous composition, or a combination thereof.

29. A method for preparing an aqueous composition comprising aluminium, a reactive compound and a protein said method comprising

- preparing or selecting an aluminium-salt that is able to provide an aqueous composition having less than 450 ppm heavy metal based on the weight of the aluminium (gram/gram) and

- combining said aluminium salt, said reactive compound, said protein and water to produce said aqueous composition.

30. A method according to claim 29, further comprising buffering said aqueous composition at a pH of between 6.5 and 8.5.

31. A method according to claim 29 or 30, further comprising packaging aliquots of said aqueous composition having less 450 ppm heavy metal based on the weight of the aluminium in the composition in separate air-tight storage containers.

32. A method for preparing a clinical grade aluminium-salt precipitate for incorporation into a medicament and/or vaccine, said method comprising preparing an aqueous solution of aluminium ions and precipitating said aluminium-ions from said solution, and determining the level of a heavy metal in the solution and/or the aluminium-salt precipitate, wherein the precipitate is selected that is able to provide an aqueous composition comprising less than 450 ppm heavy metal based on the weight of the aluminium ions (gram/gram) in the solution.

33. An aqueous composition comprising a protein and an aluminium-salt, said composition comprising less than 450 ppm heavy metal based on the weight of the aluminium in the composition.

34. An aqueous composition according to claim 33, which has been stored at temperatures higher than 20°C for at least 1 month.

35. An aqueous composition according to claim 33 or 34, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V, Cr and Mo.

36. An aqueous composition according to claim 33-35, wherein said heavy metal is selected from Cu, Ni, W, Co, Os, Ru, Cd, Ag, Fe, V.

37. An aqueous composition according to claim 33-36, wherein said heavy metal is selected from Cu or Ni.

Figure 1

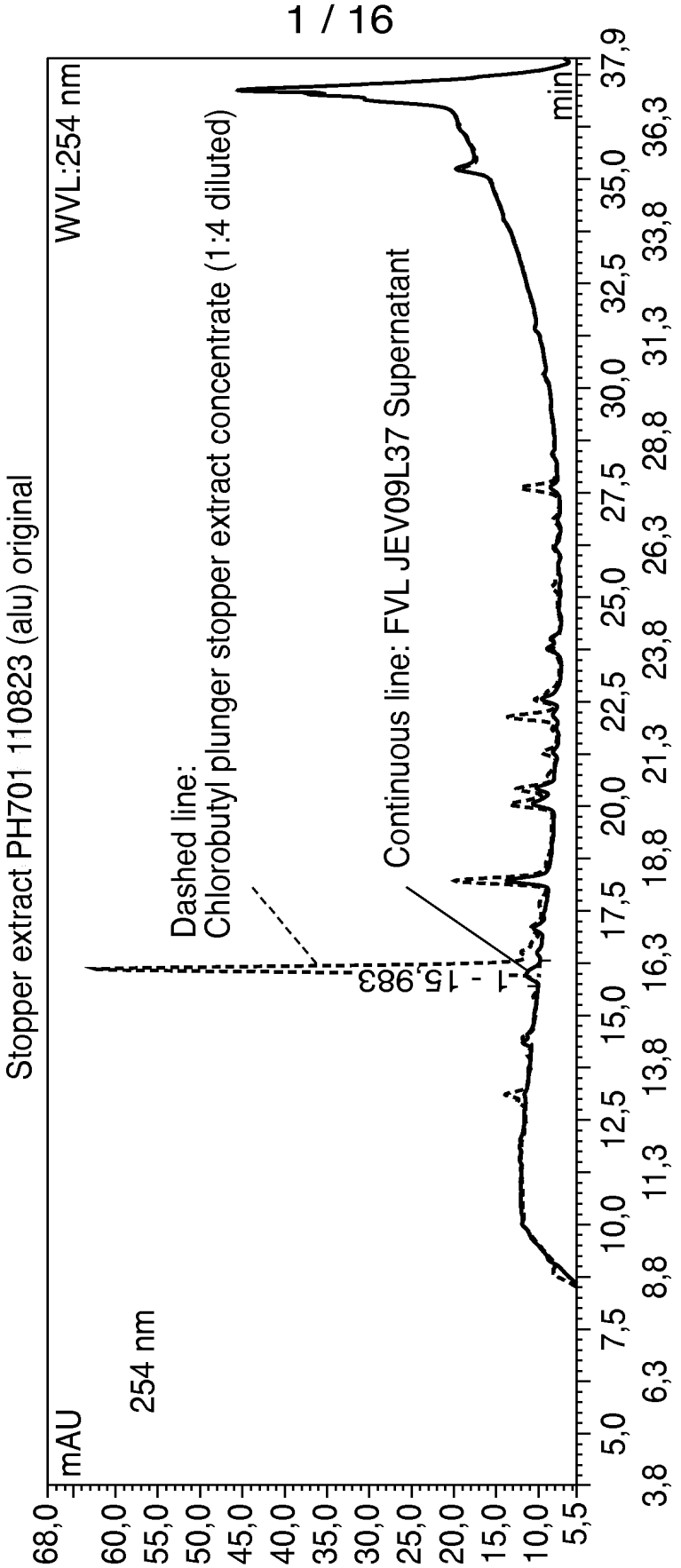

Stopper extract PH701 110823 (alu) original
WVL:254 nm
Dashed line:
Chlorobutyl plunger stopper extract concentrate (1:4 diluted)
Continuous line: FVL JEV09L37 Supernatant
mAU
254 nm
min
68,0
60,0
55,0
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,5
3,8
6,3
8,8
11,3
13,8
16,3
18,8
21,3
23,8
26,3
28,8
31,3
33,8
36,3
5,0
7,5
10,0
12,5
15,0
17,5
20,0
22,5
25,0
27,5
30,0
32,5
35,0
37,9

Figure 2

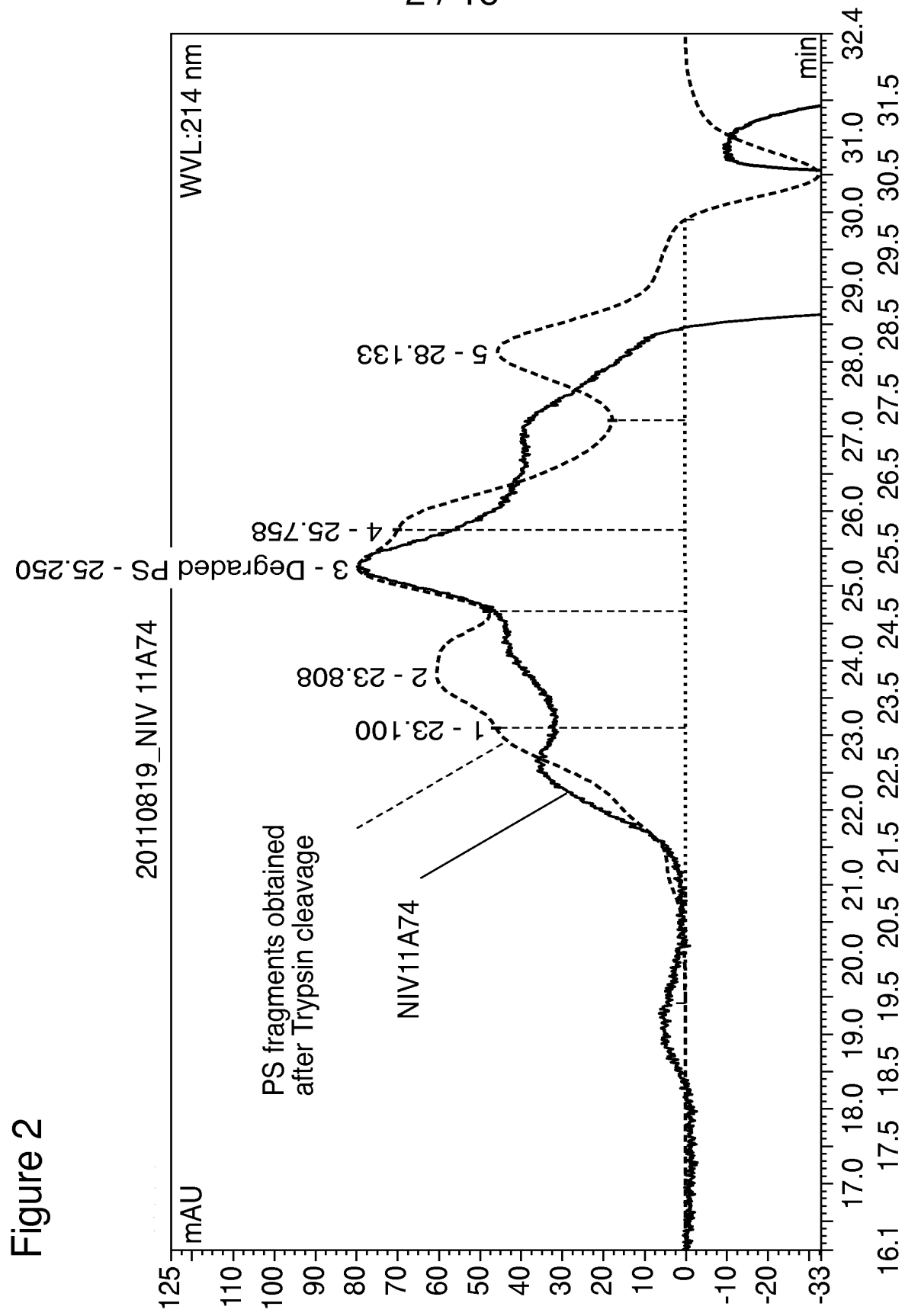

WVL:214 nm
20110819_NIV 11A74
mAU
min
PS fragments obtained after Trypsin cleavage
NIV11A74
1 - 23.100
2 - 23.808
3 - Degraded PS - 25.250
4 - 25.758
5 - 28.133

Figure 3

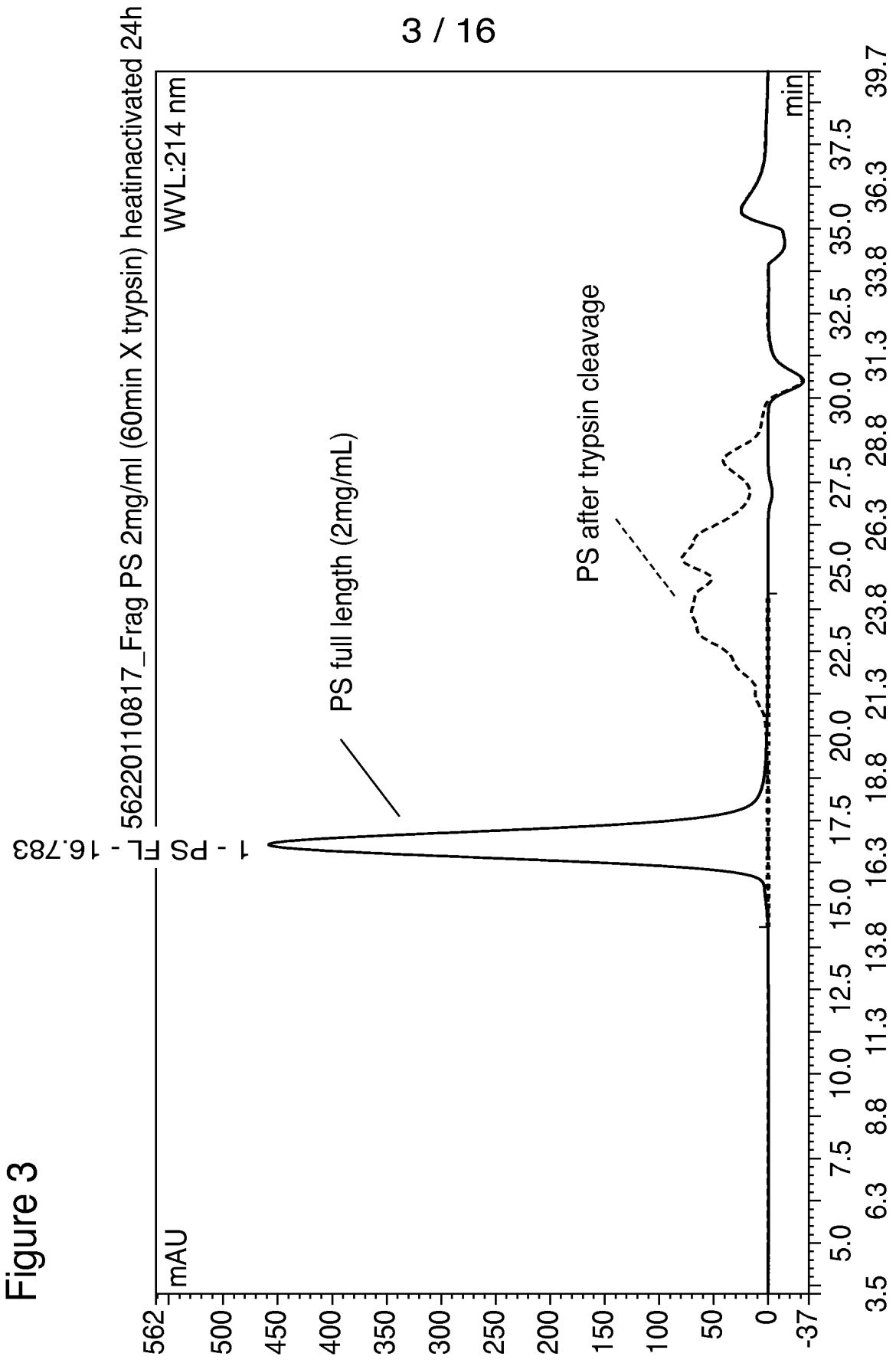

562220110817_Frag PS 2mg/ml (60min X trypsin) heatinactivated 24h
WVL:214 nm
1 - PS FL - 16.783
PS full length (2mg/mL)
PS after trypsin cleavage
mAU
min
562
500
450
400
350
300
250
200
150
100
50
0
-37
3.5
6.3
7.5
8.8
10.0
11.3
12.5
13.8
15.0
16.3
17.5
18.8
20.0
21.3
22.5
23.8
25.0
26.3
27.5
28.8
30.0
31.3
32.5
33.8
35.0
36.3
37.5
39.7

Figure 4

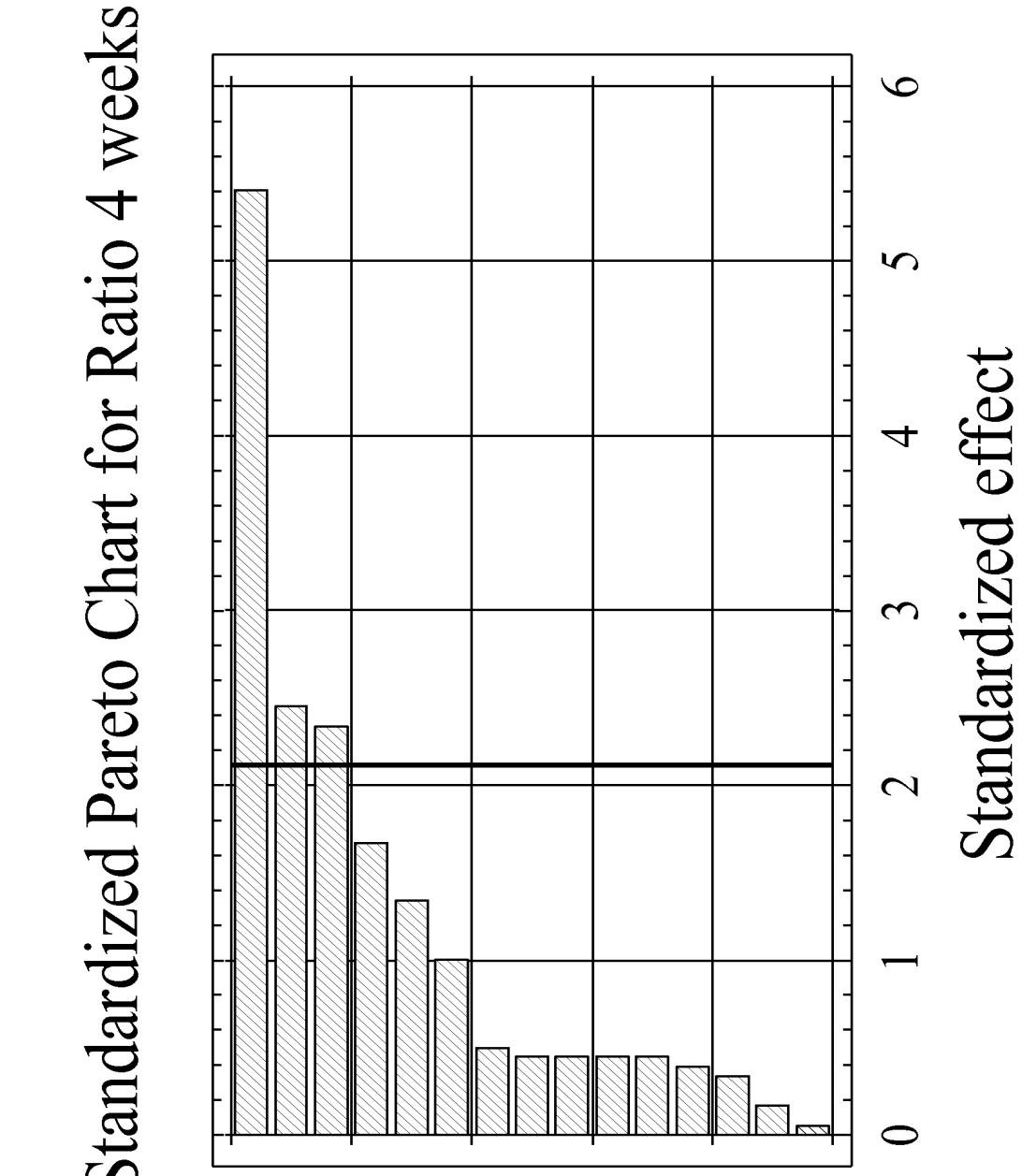

Standardized Pareto Chart for Ratio 4 weeks
B:Alum
E:Formaline
A:pH
AD
DE
AE
BD
C:PS Fragments
CE
D:Extractables
CD
BE
AC
AB
BC
0
1
2
3
4
5
6
Standardized effect

Figure 4 continued

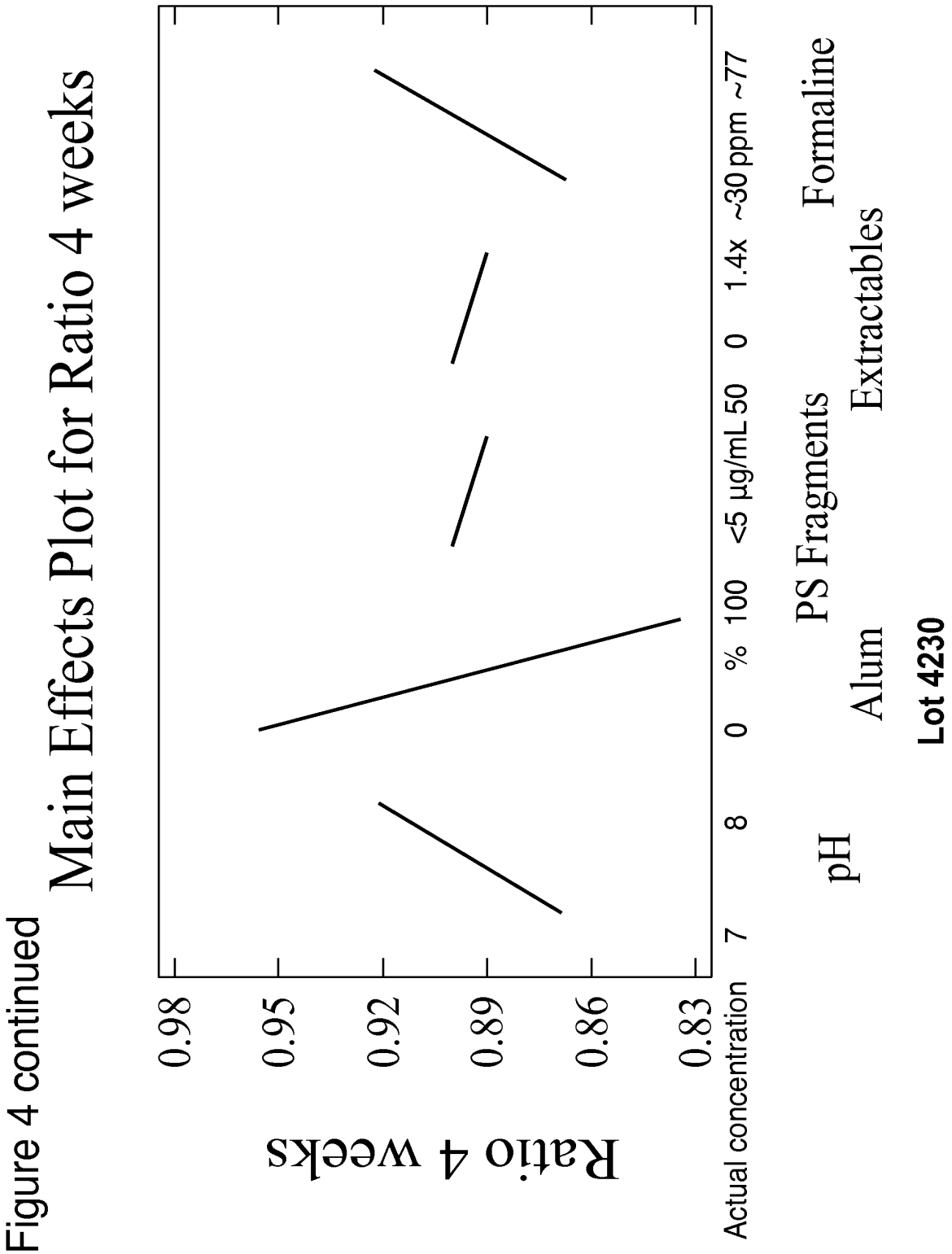


Figure 5

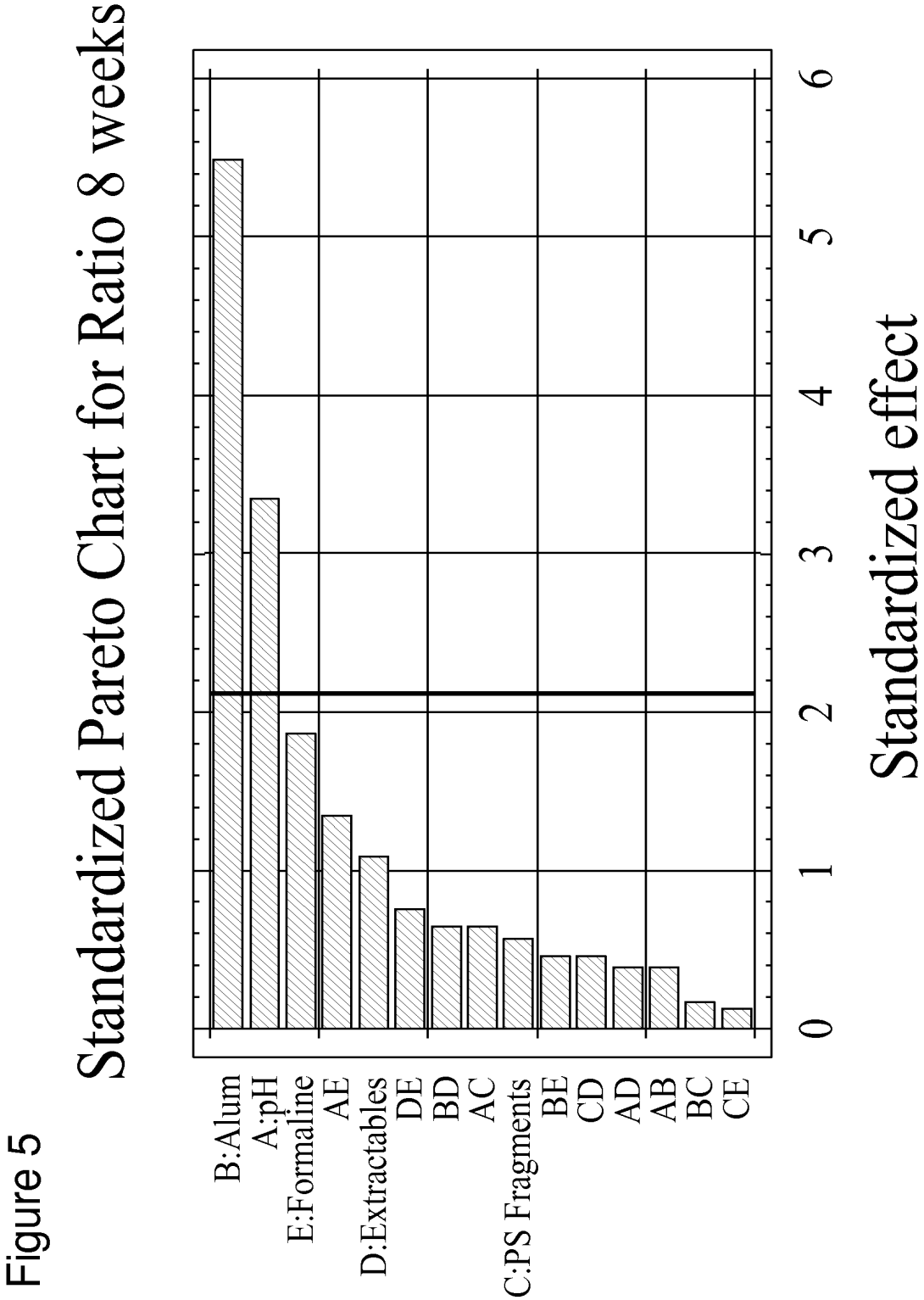

Standardized Pareto Chart for Ratio 8 weeks
B:Alum
A:pH
E:Formaline
AE
D:Extractables
DE
BD
AC
C:PS Fragments
BE
CD
AD
AB
BC
CE
0
1
2
3
4
5
6
Standardized effect

Figure 5 continued

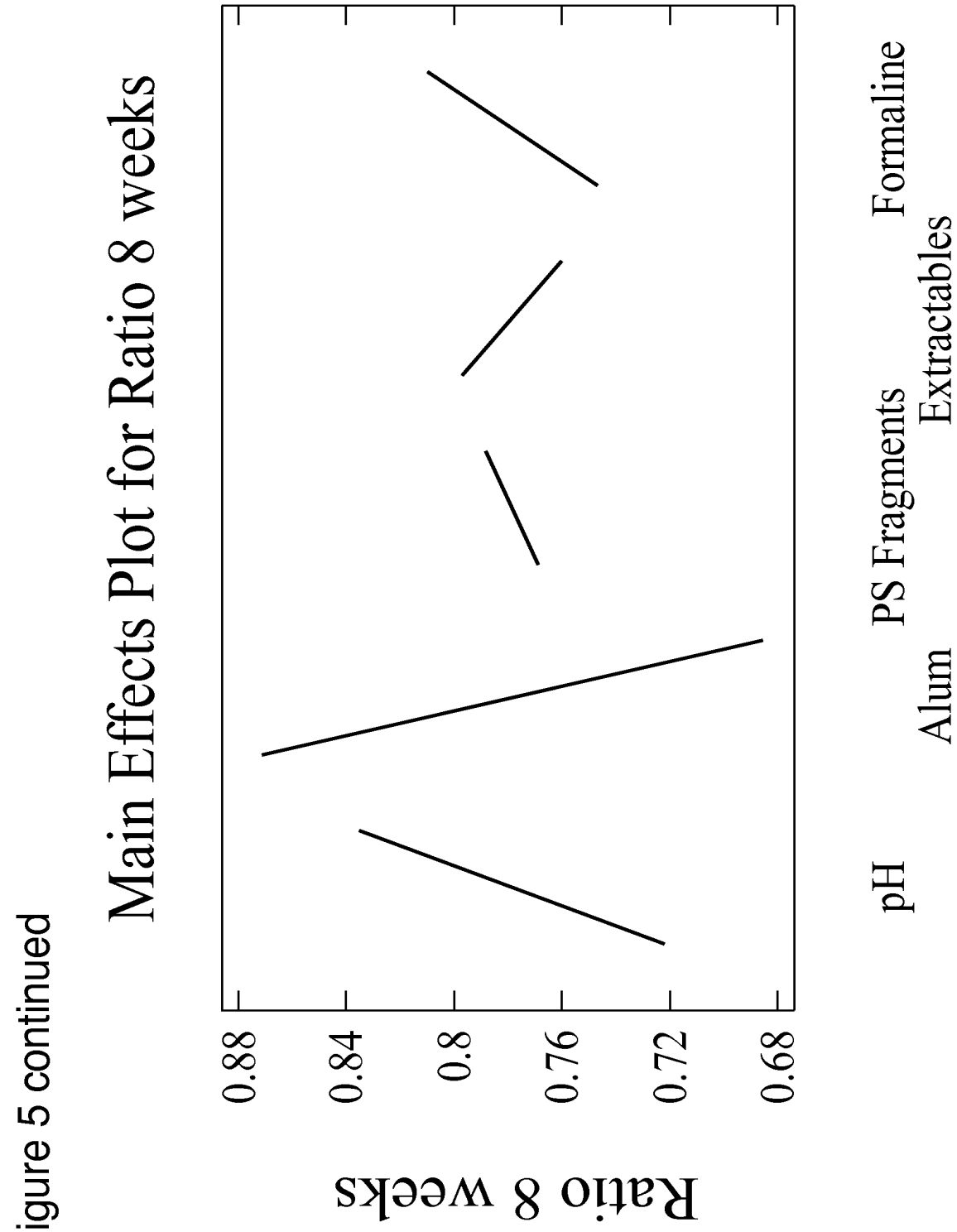


Figure 6

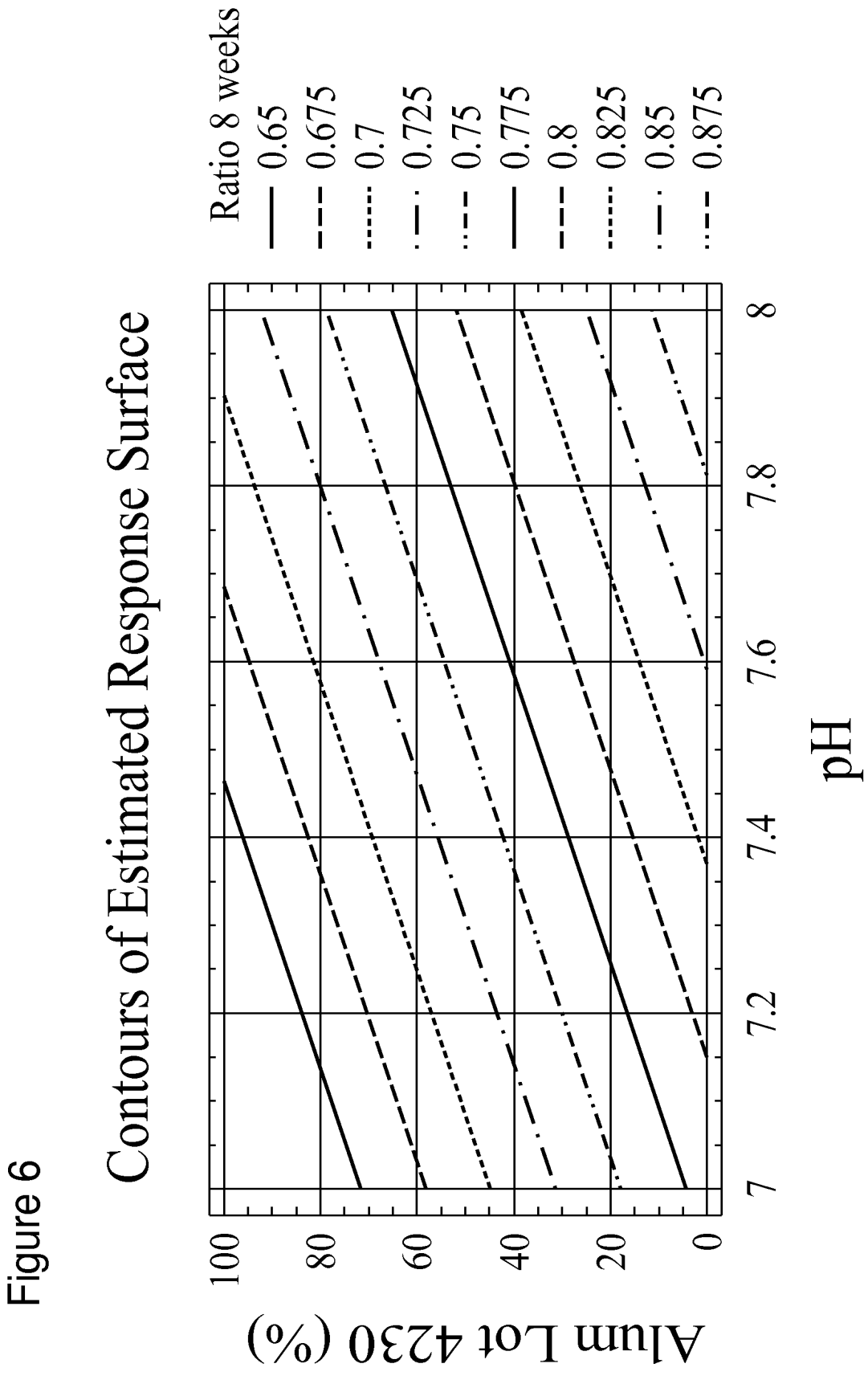

Contours of Estimated Response Surface
Ratio 8 weeks
0.65
0.675
0.7
0.725
0.75
0.775
0.8
0.825
0.85
0.875
Alum Lot 4230 (%)
100
80
60
40
20
0
7
7.2
7.4
7.6
7.8
8
pH

Figure 7

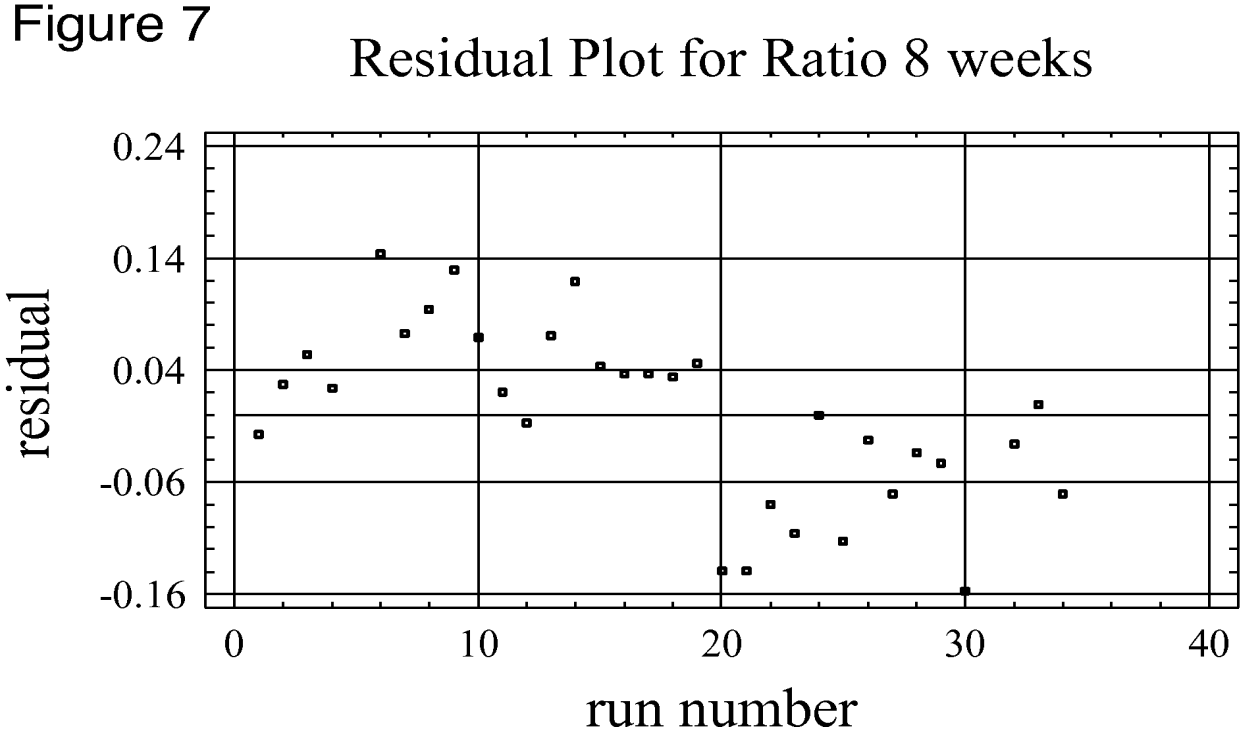
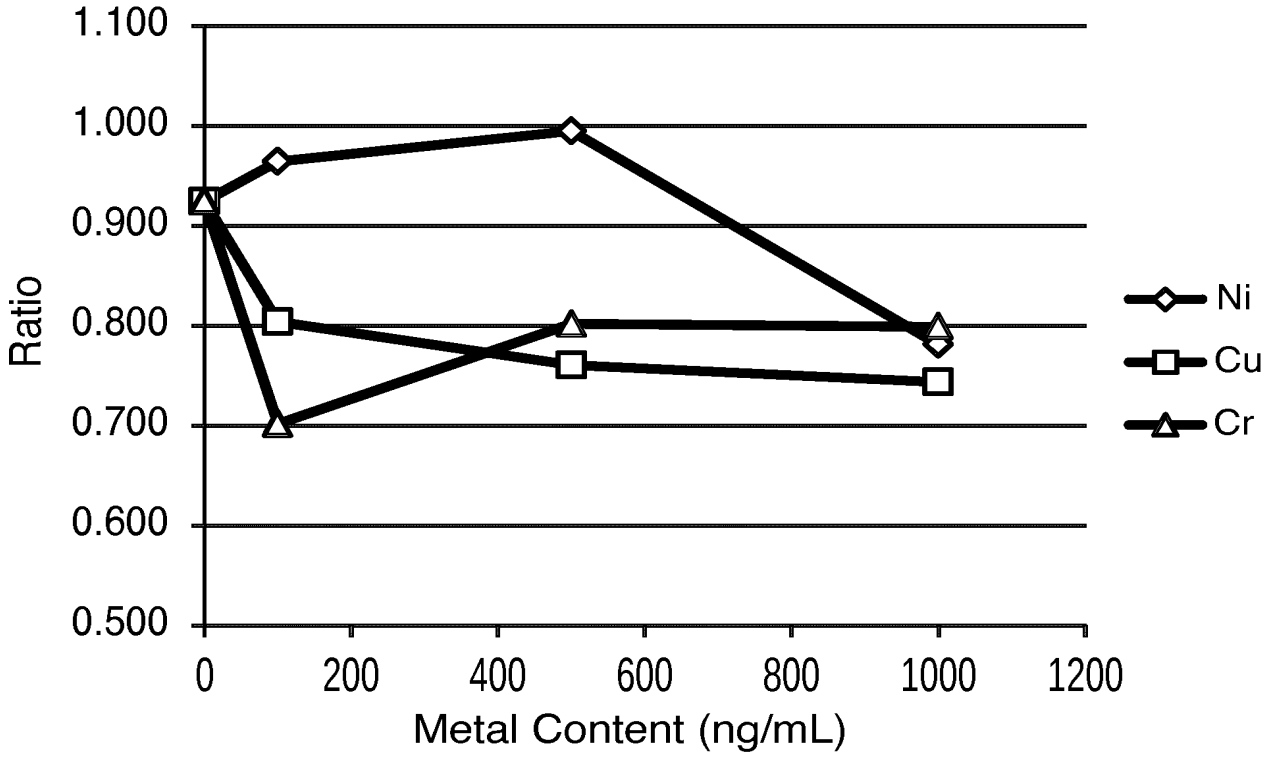


Figure 8



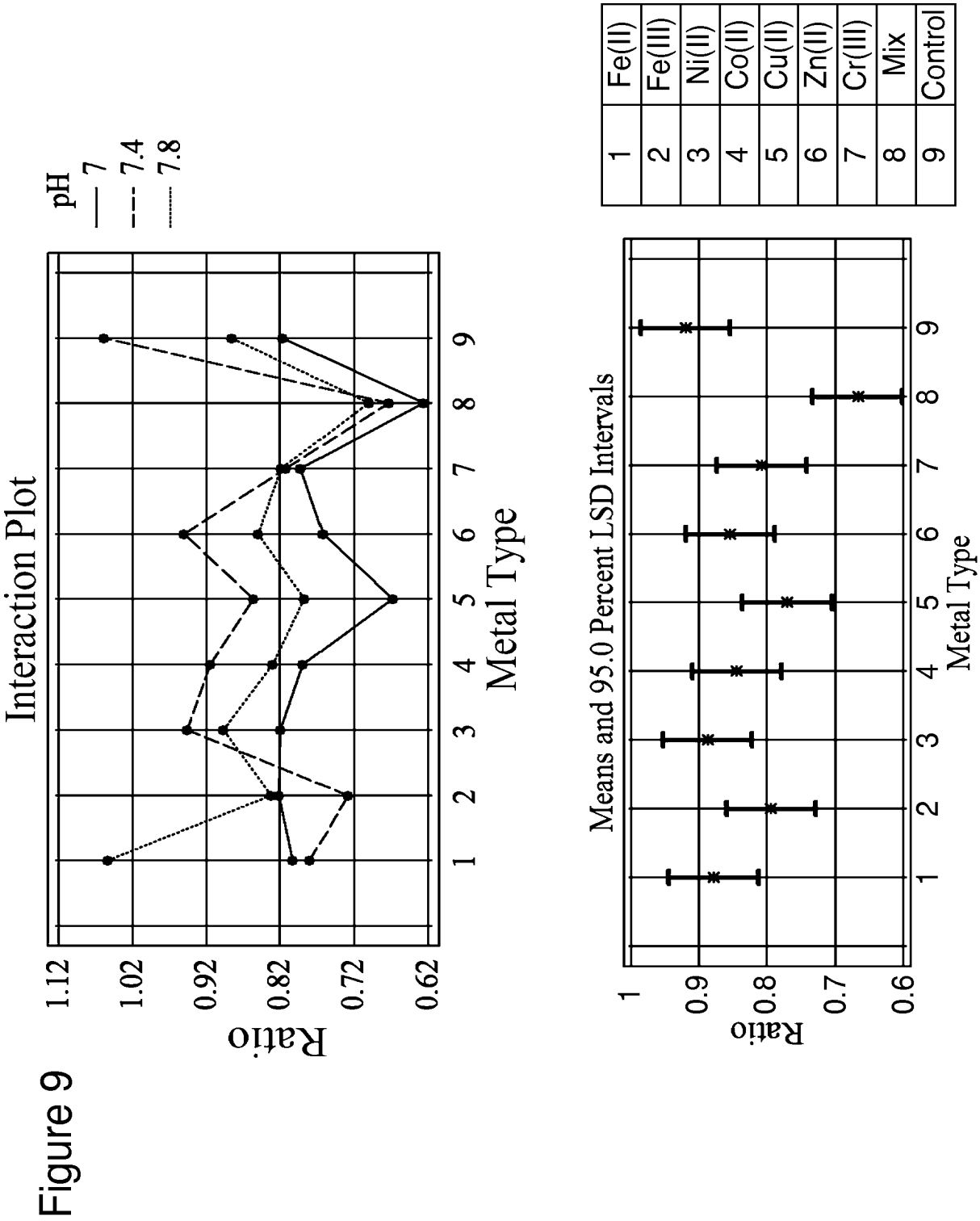

Interaction Plot
pH
7
7.4
7.8
Ratio
1.12
1.02
0.92
0.82
0.72
0.62
1 2 3 4 5 6 7 8 9
Metal Type
Means and 95.0 Percent LSD Intervals
Ratio
1
0.9
0.8
0.7
0.6
1 2 3 4 5 6 7 8 9
Metal Type
1 Fe(II)
2 Fe(III)
3 Ni(II)
4 Co(II)
5 Cu(II)
6 Zn(II)
7 Cr(III)
8 Mix
9 Control

Figure 9

Figure 9 continued

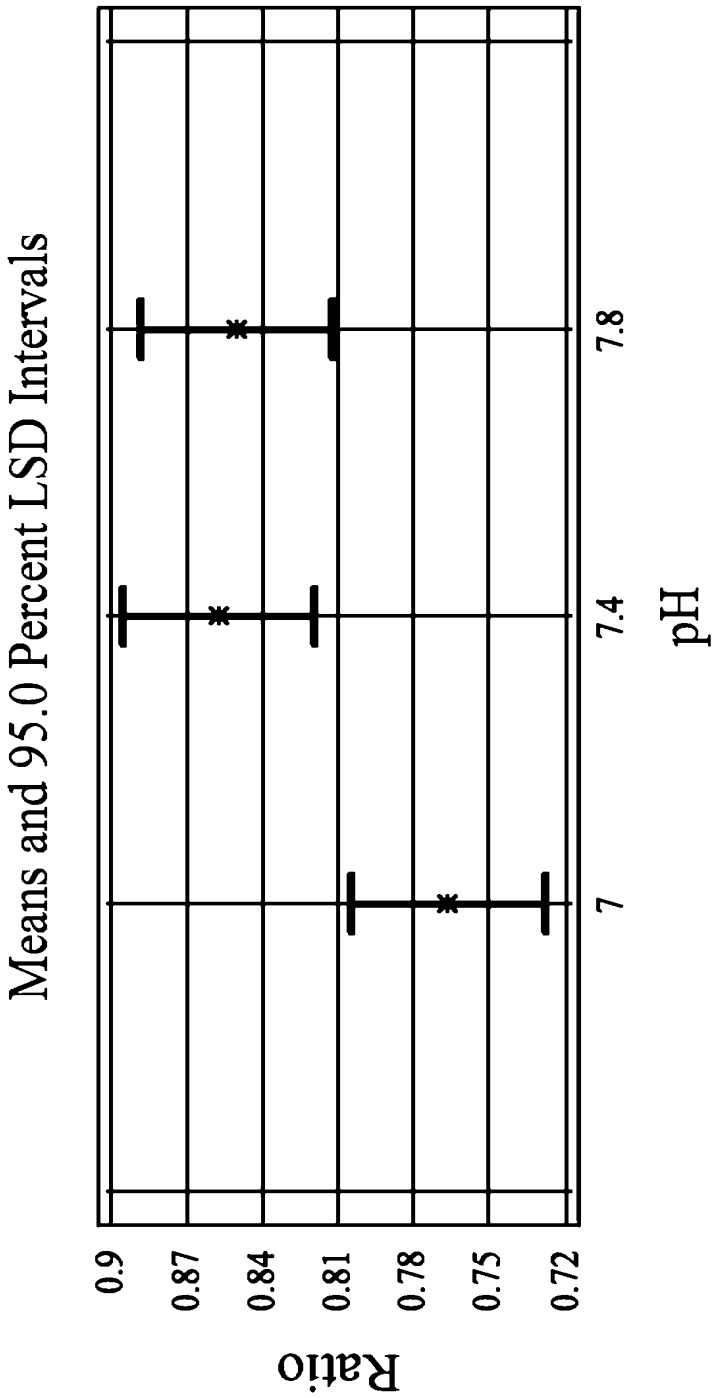
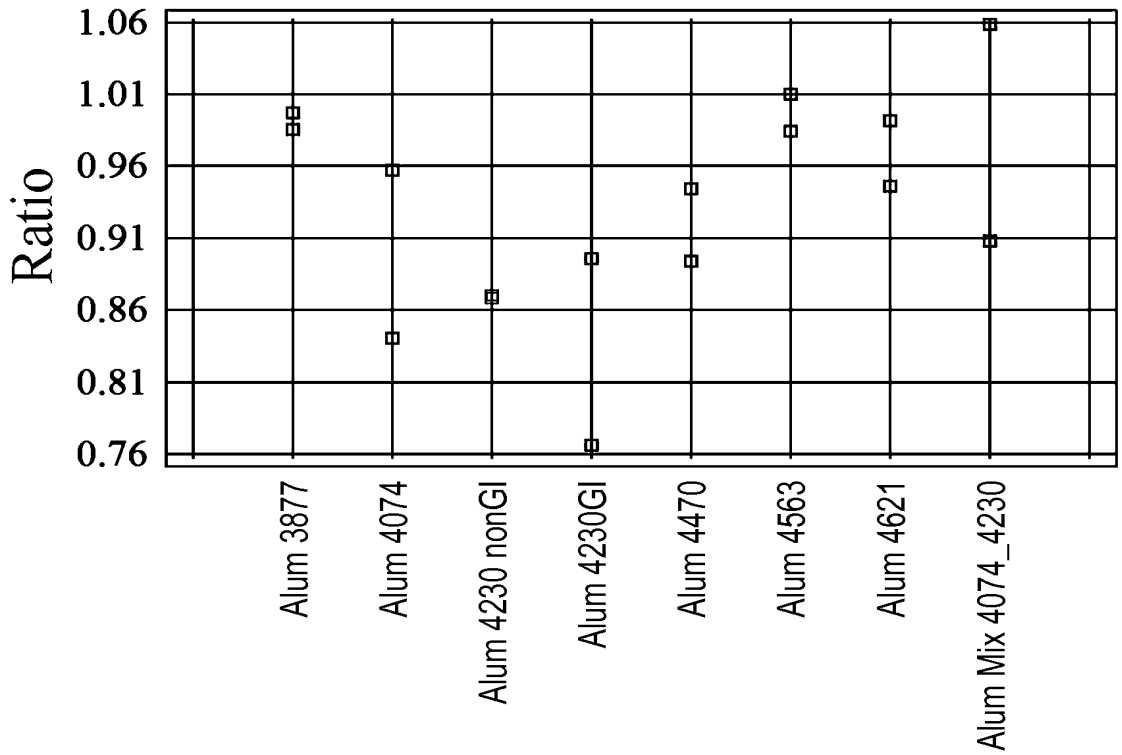


Figure 10


Scatterplot by Sample
Ratio
1.06
1.01
0.96
0.91
0.86
0.81
0.76
Alum 3877
Alum 4074
Alum 4230 nonGI
Alum 4230GI
Alum 4470
Alum 4563
Alum 4621
Alum Mix 4074_4230

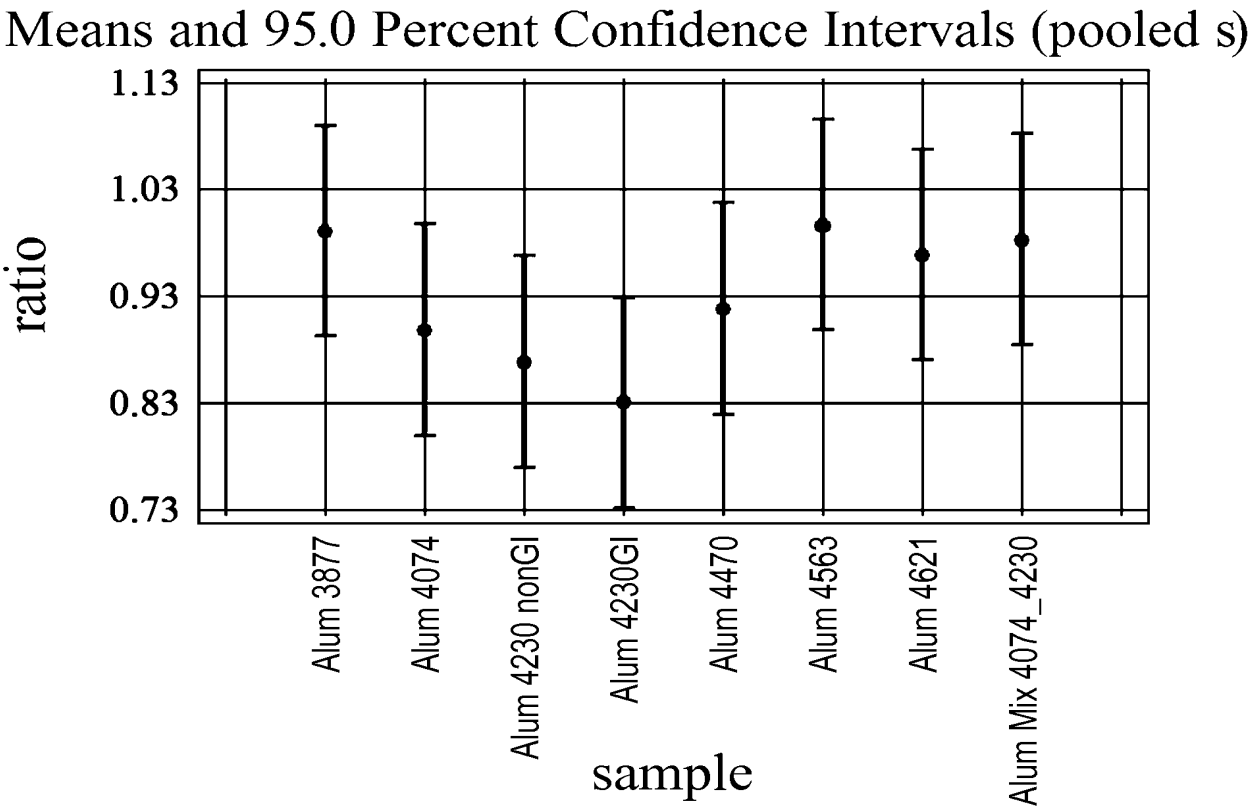

Means and 95.0 Percent Confidence Intervals (pooled s)
ratio
1.13
1.03
0.93
0.83
0.73
Alum 3877
Alum 4074
Alum 4230 nonGI
Alum 4230GI
Alum 4470
Alum 4563
Alum 4621
Alum Mix 4074_4230
sample

Figure 11

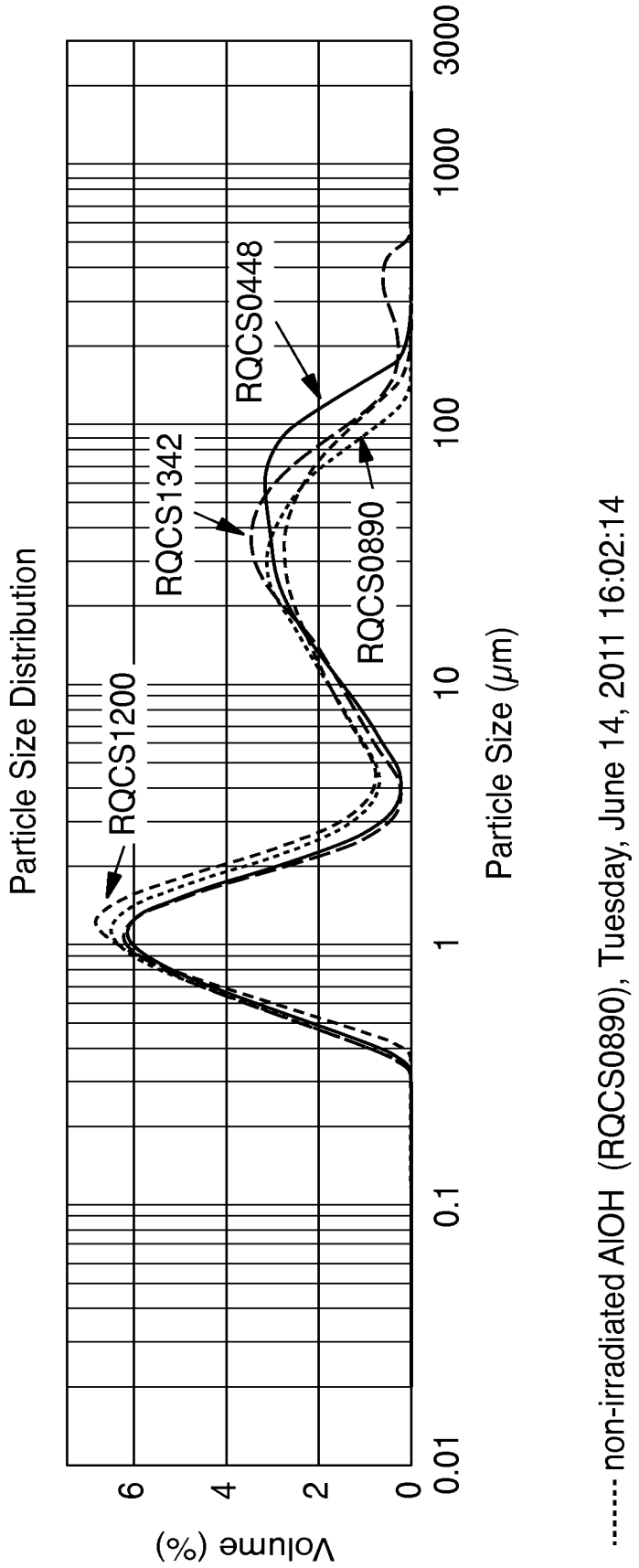

Particle Size Distribution
RQCS1200
RQCS1342
RQCS0448
RQCS0890
Volume (%)
0
2
4
6
0.01
0.1
1
10
100
1000
3000
Particle Size (μm)
non-irradiated AlOH (RQCS0890), Tuesday, June 14, 2011 16:02:14
GI AlOH (RQCS1200), Tuesday, June 14, 2011 16:10:57
GI AlOH (RQCS1342), Tuesday, June 14, 2011 16:06:52
GI AlOH (RQCS0448), Tuesday, June 14, 2011 16:15:18

Figure 12

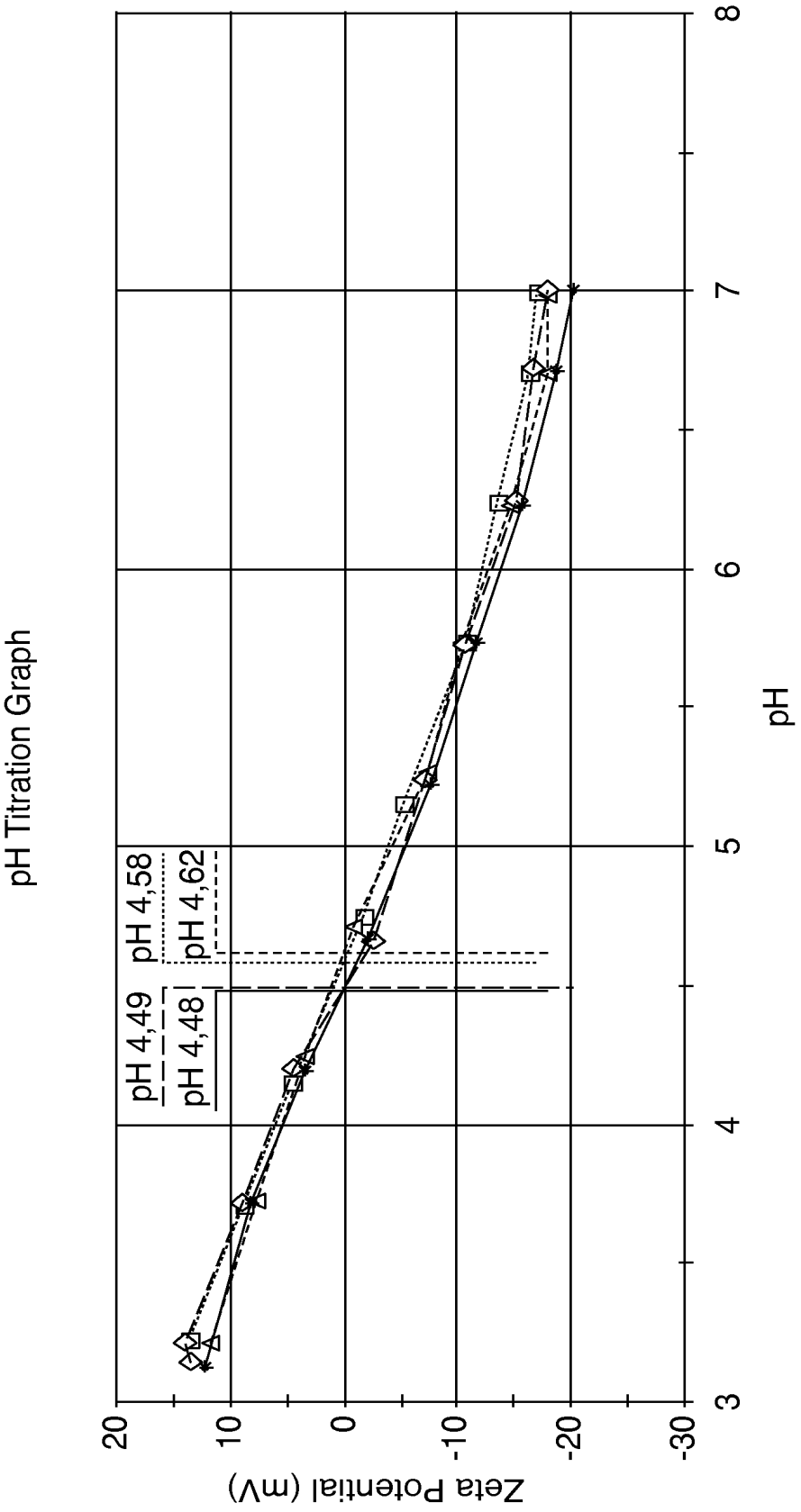

pH Titration Graph
Zeta Potential (mV)
20
10
0
-10
-20
-30
3
4
5
6
7
8
pH
pH 4,58
pH 4,62
pH 4,49
pH 4,48

Figure 13

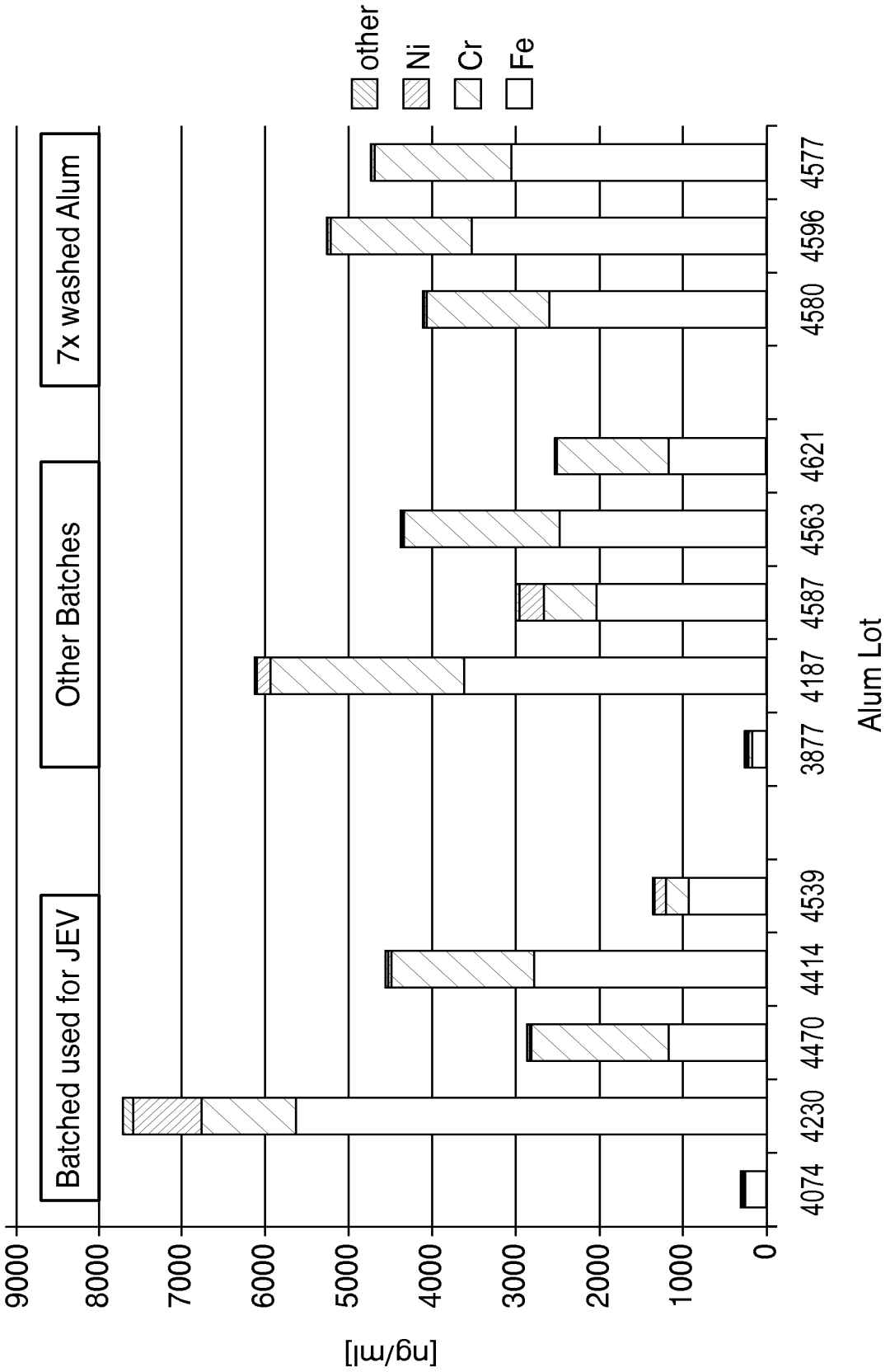

other
Ni
Cr
Fe
Batched used for JEV
Other Batches
7x washed Alum
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
[ng/ml]
4074
4230
4470
4414
4539
3877
4187
4587
4563
4621
4580
4596
4577
Alum Lot

Figure 14

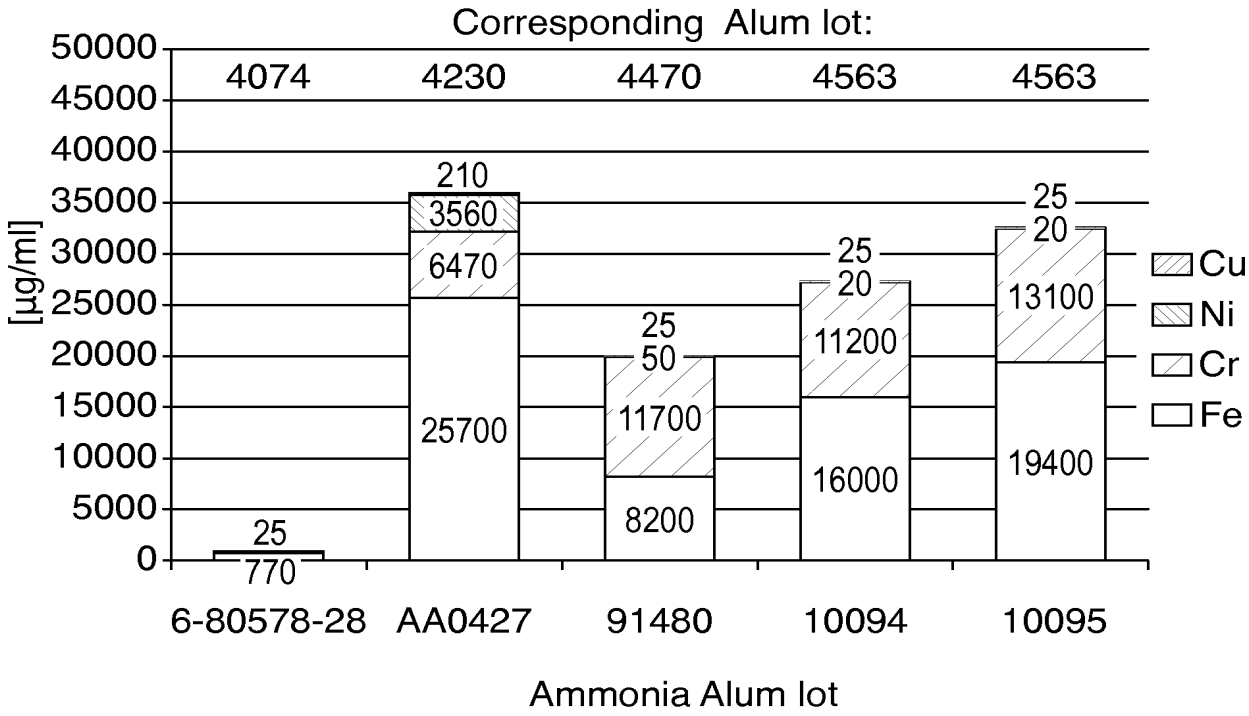


Figure 15

AHHHHHHAPAPEPVADVCSDSDNDGVCDNVDKCPDTPANVTVDANGCPAVAEVVRVQ
LDVKFDFDKSKVKENSYADIKNLADFMKQYPSTSTTVEGHTDSVGTDAYNQKLSERRAN
AVRDVLVNEYGVEGGRVNAVGYGESRPVADNATAEGRAINRRVESSHSKETEARLTAT
EDAAARAQARADEAYRKADEALGAAQKAQQTADEANERALRMLEKASRK

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/074701

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 A61K39/39
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KULAMANI DASH ET AL: "Sample Treatment Approaches for Trace Level Determination of Cesium in Hepatitis B Vaccine by Suppressed Ion Chromatography", CHROMATOGRAPHIA ; AN INTERNATIONAL JOURNAL FOR RAPID COMMUNICATION IN CHROMATOGRAPHY, ELECTROPHORESIS AND ASSOCIATED TECHNIQUES, vol. 75, no. 1 - 2, 8 November 2011 (2011-11-08), pages 17-23, XP019993899, VIEWEG VERLAG, WI ISSN: 1612-1112, DOI: 10.1007/S10337-011-2161-9 page 19, right-hand column, last paragraph - page 20, left-hand column, paragraph 1 page 20, right-hand column, paragraph 1 - paragraph 2; table 3 ----- -/--	5,7,8, 10,11, 15-17, 20,21, 26-28, 33,35,36

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2013	18/02/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Montero Lopez, B

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SRIVASTAVA A K ET AL: "A purified inactivated Japanese encephalitis virus vaccine made in vero cells", VACCINE, vol. 19, no. 31, 14 August 2001 (2001-08-14), pages 4557-4565, XP027321987, ELSEVIER LTD, GB ISSN: 0264-410X [retrieved on 2001-08-14] abstract page 4560, left-hand column, paragraph 2 page 4561, right-hand column, last paragraph - page 4562, left-hand column, paragraph 1 page 4563, right-hand column, last paragraph; table 5 page 4564, right-hand column, last paragraph	1-3,5-37
X	ANONYMOUS: "Rehydragel Adjuvants. Product profile", General Chemical , 2008, pages 1-2, XP002684848, Retrieved from the Internet: URL:http://www.generalchemical.com/assets/pdf/Rehydragel_Adjuvants_Product_Profile.pdf [retrieved on 2012-10-08]	4,27,29, 32
A	the whole document	1-3,5-26
A	WO 2009/158284 A2 (MAYO FOUNDATION [US]; BARRY MICHAEL A [US]) 30 December 2009 (2009-12-30) page 5, line 1 - line 19	1-37
A	LINDBLAD, EB: "Special feature. Aluminium compounds for use in vaccines", IMMUNOL. AND CELL BIOL., vol. 82, 2004, pages 497-505, XP008151421, cited in the application the whole document	1-37

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI S; SCHONEICH C; BORCHARDT RT: "Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization.", BIOTECHNOL BIOENG., vol. 48, no. 5, 5 December 1995 (1995-12-05), pages 490-500, XP055040145, cited in the application abstract page 491, left-hand column, paragraph 2 - paragraph 3 page 497, right-hand column, paragraph 3 -----	1-37
A	TIA ESTEY ET AL: "Evaluation of chemical degradation of a trivalent recombinant protein vaccine against botulinum neurotoxin by LysC peptide mapping and MALDI-TOF mass spectrometry", JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 98, no. 9, 1 September 2009 (2009-09-01), pages 2994-3012, XP055040203, ISSN: 0022-3549, DOI: 10.1002/jps.21543 abstract page 2995, left-hand column, paragraph 1 - right-hand column, paragraph 1 page 2996, right-hand column, paragraph 2 - page 2997, left-hand column, paragraph 4 -----	1-37
A	US 2005/158334 A1 (CONTORNI MARIO [IT] ET AL) 21 July 2005 (2005-07-21) the whole document -----	1-37

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/074701

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009158284 A2	30-12-2009	US 2011189217 A1	04-08-2011
		WO 2009158284 A2	30-12-2009
US 2005158334 A1	21-07-2005	AT 448794 T	15-12-2009
		BR 0211437 A	13-07-2004
		DK 1409013 T3	18-01-2010
		EP 2168597 A1	31-03-2010
		EP 2255827 A1	01-12-2010
		EP 2266605 A1	29-12-2010
		ES 2334495 T3	11-03-2010
		JP 4509554 B2	21-07-2010
		JP 2004538291 A	24-12-2004
		JP 2009173681 A	06-08-2009
		MX PA04000753 A	17-02-2005
		PT 1409013 E	26-02-2010
		US 2005158334 A1	21-07-2005
		US 2008160045 A1	03-07-2008

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(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
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(51) International Patent Classification:
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(21) International Application Number:
PCT/US2016/021696

(22) International Filing Date:
10 March 2016 (10.03.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/131,326 11 March 2015 (11.03.2015) US

(71) Applicant: **THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY OF THE ARMY, ON BEHALF OF THE WALTER REED ARMY INSTITUTE OF RESEARCH** [US/US]; 504 Scott Street, U.S. Army Medical Research and Materiel Command, Fort Detrick, Maryland 21702-5012 (US).

(72) Inventors; and
(71) Applicants : **THOMAS, Stephen J.** [US/US]; 503 Robert Grant Avenue, c/o Walter Reed Army Institute of Research, Viral Diseases Branch, Silver Spring, Maryland 20910 (US). **ECKELS, Kenneth H.** [US/US]; 503 Robert Grant Avenue, c/o Walter Reed Army Institute of Research, Viral Diseases Branch, Silver Spring, Maryland 20910 (US). **PUTNAK, Joseph R.** [US/US]; 503 Robert Grant Avenue, c/o Walter Reed Army Institute of Research, Viral Diseases Branch, Silver Spring, Maryland 20910 (US).

(74) Agent: **SUNDBY, Suzannah K.**; 1050 30th Street, NW, Canady + Lortz LLP, Washington, District of Columbia 20007 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*

(54) **Title**: COMBINATION PURIFIED INACTIVATED VACCINE FOR FLAVIVIRUSES

(57) **Abstract**: Disclosed herein are immunogenic compositions comprising one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and methods of making and using thereof.

WO 2016/145149 A1

COMBINATION PURIFIED INACTIVATED VACCINES FOR FLAVIVIRUSES

[0001] ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made by employees of the United States Army Medical Research and Materiel Command, which is an agency of the United States Government. The Government has certain rights in the invention.

[0003] BACKGROUND OF THE INVENTION

[0004] 1. FIELD OF THE INVENTION

[0005] The present invention relates to immunogenic compositions which comprise at least one inactivated dengue virus and at least one inactivated non-dengue flavivirus, and methods of making and using thereof.

[0006] 2. DESCRIPTION OF THE RELATED ART

[0007] Flaviviruses are enveloped, single-stranded, positive-sense RNA viruses in the genus *Flavivirus,* family *Flaviviridae*. The viral RNA genome is approximately 11 kb in length and contains one long open reading frame (ORF) encoding four virion structural proteins, capsid (C), membrane (M), its precursor pre-membrane (prM), and envelope (E), and 7 non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, flanked by 5'- and 3'-untranslated regions (5'UTR/3'UTR), approximately 100 and 450 nucleotides (nt) long, respectively. All viral proteins derive from the post-translational processing of a polyprotein precursor by both virally-encoded and host proteinases. The C protein comprises the virion core, which encapsidates the viral RNA genome. The prM, M, and E proteins are integral membrane proteins within the virion envelope. In completely matured, infectious virions the E protein exists as an N-glycosylated dimer, 180 copies per virion, along with an equal number of M protein molecules. The NS proteins, although they are not incorporated into the virion, play critical roles in the virus life cycle, including proteolytic processing, RNA replication, virion maturation, and the modulation of host cell functions and activities such as those involved in interferon and apoptosis pathways.

[0008] There are nearly 70 known flaviviruses, 30 of which have been associated with human diseases of varying incidence and severity. The flaviviruses were originally grouped and typed by their reactivity in serological assays (i.e., virus-neutralization, hemagglutination, and complement-fixation) according to their unique (virus specific) or shared (cross-reactive) antigenic determinants. These groupings were later confirmed and extended by monoclonal antibody mapping of discrete epitopic determinants and genetic sequence analysis of the viral RNAs. Arguably, the most medically important flaviviruses

in terms of disease incidence and severity are the arthropod-borne viruses or Arboviruses, particularly those in Group B, which are transmitted by mosquito or tick vectors. These include the dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV), Zika virus (ZIKV), St. Louis encephalitis virus (SLEV), Tick-borne encephalitis virus (TBEV) (including the Russian Spring Summer encephalitis virus (RSSEV)).

[0009] The disease burden from flavivirus infections is enormous and growing, especially considering that accurate estimates of incidence are hampered by asymptomatic disease, underreporting, and limited diagnostic capabilities in many endemic areas. For example, current estimates suggest that more than 3.6 billion people may be at risk for infection by DENV in the tropics and subtropics in both the eastern and western hemispheres where the primary mosquito vector *Aedes aegypti* is prevalent. It is estimated nearly 400 million infections by DENV occur each year with over 95 million being symptomatic and 2 million severe.

[0010] Individuals, including United States military personnel, are at significant risk for infection by flaviviruses, such as YFV, JEV, DENV, and ZIKV when stationed or travelling in endemic areas and those living in North America are at risk of infection by WNV. The Southern United States has mosquito vectors capable of transmitting flaviviruses as evidence by the annual occurrence of DENV transmission in Florida and Texas. There are existing licensed (U.S. and foreign) live attenuated YFV and inactivated JEV and Tick-Borne Encephalitis (TBE) vaccines and live attenuated chimeric vaccines based on ChimeriVax® technology against JEV, DENV, and WNV. There has yet to be developed a vaccine for ZIKV. Most inactivated and attenuated flavivirus vaccines against a given flavivirus require a burdensome 2 to 3 dose primary immunization regimen which requires completion about one month prior to exposure to the flavivirus. In the case of DENV ChimeriVax®, the immunization schedule spans one year (dosing 0, 6, and 12 months). The inactivated JEV vaccine (IXIARO®) requires at least 2 doses for protective immunization.

[0011] Although some flaviviruses are restricted to specific regions of the world, many are more widespread and there are significant overlaps. For example, in Africa and the Americas both DENV and YFV are present, whereas in larger parts of Asia, DENV and JEV are endemic. ZIKV is now actively transmitted in Asia, Africa, and the Americas. Thus, before traveling to places like Africa and Asia, one must undergo lengthy, multiple immunizations regimens.

[0012] Therefore, a need exists for a single vaccine composition that effectively protects against multiple, different flaviviruses.

[0013] SUMMARY OF THE INVENTION

[0014] The present invention provides immunogenic compositions comprising one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle. The present invention provides immunogenic compositions consisting essentially of one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle. The present invention provides immunogenic compositions consisting of one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle. In some embodiments, the one or more inactivated dengue viruses and the one or more inactivated non-dengue flaviviruses are each provided in an immunogenically effective amount. In some embodiments, some or all of the inactivated viruses are purified. In some embodiments, the one or more inactivated dengue viruses are selected from the group consisting of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses consist of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses comprise or consist of 1, 2, 3, or 4 DENV serotypes. In some embodiments, the dengue virus is an inactivated DENV-1 produced from deposited DENV-1 strain having ATCC accession number VR-2649. In some embodiments, the dengue virus is an inactivated DENV-2 produced from deposited DENV-2 strain having ATCC accession number VR-2650. In some embodiments, the dengue virus is an inactivated DENV-3 produced from deposited DENV-3 strain having ATCC accession number VR-2654. In some embodiments, the dengue virus is an inactivated DENV-4 produced from deposited DENV-4 strain having ATCC accession number VR-2651. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever group viruses and Japanese encephalitis group viruses. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). In some embodiments, the one or more inactivated non-dengue flaviviruses are yellow fever virus (YFV) and Japanese encephalitis virus (JEV). In some embodiments, the one or more inactivated non-dengue flavivirus includes Zika virus (ZIKV). In some embodiments, the

one or more inactivated non-dengue flavivirus is Zika virus (ZIKV). In some embodiments, the one or more inactivated dengue viruses are present in an immunologically effective amount and/or the one or more inactivated non-dengue flaviviruses are present in an immunologically effective amount. In some embodiments, each of the one or more inactivated dengue viruses are present in an immunologically effective amount. In some embodiments, the one or more inactivated dengue viruses are purified and/or the one or more inactivated non-dengue flaviviruses are purified. In some embodiments, the immunogenic composition further comprises an adjuvant. In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and IXIARO® vaccine (Intercell USA, Inc.). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV, XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare), and IXIARO® vaccine (Intercell USA, Inc.).

[0015] In some embodiments, the present invention provides methods of producing antibodies against a dengue virus and a non-dengue flavivirus in a subject, which comprises administering to the subject an immunogenic composition comprising, consisting essentially of, or consisting of one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle. In some embodiments, the one or more inactivated dengue viruses and the one or more inactivated non-dengue flaviviruses are each provided in an immunogenically effective amount. In some embodiments, some or all of the inactivated viruses are purified. In some embodiments, the one or more inactivated dengue viruses are selected from the group consisting of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses consist of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses comprise or consist of 1, 2, 3, or 4 DENV serotypes. In some embodiments, the dengue virus is an inactivated DENV-1 produced from deposited DENV-1 strain having ATCC accession number VR-2649. In some embodiments, the dengue virus is an inactivated DENV-2 produced from deposited DENV-2 strain having ATCC accession number VR-2650. In some embodiments, the dengue virus is an inactivated DENV-3 produced from deposited DENV-3 strain having ATCC accession number VR-2654. In some embodiments, the dengue virus is an inactivated DENV-4

produced from deposited DENV-4 strain having ATCC accession number VR-2651. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever group viruses and Japanese encephalitis group viruses. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). In some embodiments, the one or more inactivated non-dengue flaviviruses are yellow fever virus (YFV) and Japanese encephalitis virus (JEV). In some embodiments, the one or more inactivated non-dengue flavivirus includes Zika virus (ZIKV). In some embodiments, the one or more inactivated non-dengue flavivirus is Zika virus (ZIKV). In some embodiments, the one or more inactivated dengue viruses are present in an immunologically effective amount and/or the one or more inactivated non-dengue flaviviruses are present in an immunologically effective amount. In some embodiments, each of the one or more inactivated dengue viruses are present in an immunologically effective amount. In some embodiments, the one or more inactivated dengue viruses are purified and/or the one or more inactivated non-dengue flaviviruses are purified. In some embodiments, the immunogenic composition further comprises an adjuvant. In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and IXIARO® vaccine (Intercell USA, Inc.). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV, XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare), and IXIARO® vaccine (Intercell USA, Inc.). In some embodiments, the immunogenic composition is administered as a single dose on a 0-28 day vaccination schedule. In some embodiments, the immunogenic composition is administered by intramuscular injection. In some embodiments, the subject is a non-human animal, such as rodents and non-human primates, which may or may not be laboratory test animals. In some embodiments, the subject is a human. In some embodiments, the subject is at risk of being exposed to one or more flaviviruses.

[0016] In some embodiments, the present invention provides medicaments for producing antibodies against a dengue virus and a non-dengue flavivirus in a subject, which comprises an immunogenic composition comprising, consisting essentially of, or consisting of one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle. In some embodiments, the one or more inactivated dengue viruses and the one or more inactivated non-dengue

flaviviruses are each provided in an immunogenically effective amount. In some embodiments, some or all of the inactivated viruses are purified. In some embodiments, the one or more inactivated dengue viruses are selected from the group consisting of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses consist of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses comprise or consist of 1, 2, 3, or 4 DENV serotypes. In some embodiments, the dengue virus is an inactivated DENV-1 produced from deposited DENV-1 strain having ATCC accession number VR-2649. In some embodiments, the dengue virus is an inactivated DENV-2 produced from deposited DENV-2 strain having ATCC accession number VR-2650. In some embodiments, the dengue virus is an inactivated DENV-3 produced from deposited DENV-3 strain having ATCC accession number VR-2654. In some embodiments, the dengue virus is an inactivated DENV-4 produced from deposited DENV-4 strain having ATCC accession number VR-2651. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever group viruses and Japanese encephalitis group viruses. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). In some embodiments, the one or more inactivated non-dengue flaviviruses are yellow fever virus (YFV) and Japanese encephalitis virus (JEV). In some embodiments, the one or more inactivated non-dengue flavivirus includes Zika virus (ZIKV). In some embodiments, the one or more inactivated non-dengue flavivirus is Zika virus (ZIKV). In some embodiments, the one or more inactivated dengue viruses are present in an immunologically effective amount and/or the one or more inactivated non-dengue flaviviruses are present in an immunologically effective amount. In some embodiments, each of the one or more inactivated dengue viruses are present in an immunologically effective amount. In some embodiments, the one or more inactivated dengue viruses are purified and/or the one or more inactivated non-dengue flaviviruses are purified. In some embodiments, the immunogenic composition further comprises an adjuvant. In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and IXIARO® vaccine (Intercell USA, Inc.). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV, XRX-001

Yellow Fever 17D inactivated vaccine (GE Healthcare), and IXIARO® vaccine (Intercell USA, Inc.). In some embodiments, the subject is a non-human animal, such as rodents and non-human primates, which may or may not be laboratory test animals. In some embodiments, the subject is a human. In some embodiments, the subject is at risk of being exposed to one or more flaviviruses.

[0017] In some embodiments, the present invention is directed to the use of an immunogenic composition comprising, consisting essentially of, or consisting of one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle. In some embodiments, the one or more inactivated dengue viruses and the one or more inactivated non-dengue flaviviruses are each provided in an immunogenically effective amount. In some embodiments, some or all of the inactivated viruses are purified. In some embodiments, the one or more inactivated dengue viruses are selected from the group consisting of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses consist of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4. In some embodiments, the one or more inactivated dengue viruses comprise or consist of 1, 2, 3, or 4 DENV serotypes. In some embodiments, the dengue virus is an inactivated DENV-1 produced from deposited DENV-1 strain having ATCC accession number VR-2649. In some embodiments, the dengue virus is an inactivated DENV-2 produced from deposited DENV-2 strain having ATCC accession number VR-2650. In some embodiments, the dengue virus is an inactivated DENV-3 produced from deposited DENV-3 strain having ATCC accession number VR-2654. In some embodiments, the dengue virus is an inactivated DENV-4 produced from deposited DENV-4 strain having ATCC accession number VR-2651. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever group viruses and Japanese encephalitis group viruses. In some embodiments, the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). In some embodiments, the one or more inactivated non-dengue flaviviruses are yellow fever virus (YFV) and Japanese encephalitis virus (JEV). In some embodiments, the one or more inactivated non-dengue flavivirus includes Zika virus (ZIKV). In some embodiments, the one or more inactivated non-dengue flavivirus is Zika virus (ZIKV). In some embodiments, the one or more inactivated dengue viruses are present in an immunologically effective amount and/or the one or more inactivated non-dengue

flaviviruses are present in an immunologically effective amount. In some embodiments, each of the one or more inactivated dengue viruses are present in an immunologically effective amount. In some embodiments, the one or more inactivated dengue viruses are purified and/or the one or more inactivated non-dengue flaviviruses are purified. In some embodiments, the immunogenic composition further comprises an adjuvant. In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV and IXIARO® vaccine (Intercell USA, Inc.). In some embodiments, the immunogenic composition is a mixture of one or more inactivated DENV, XRX-001 Yellow Fever 17D inactivated vaccine (GE Healthcare), and IXIARO® vaccine (Intercell USA, Inc.).

[0018] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

[0019] DETAILED DESCRIPTION OF THE INVENTION

[0020] The present invention relates to immunogenic compositions which comprise at least one inactivated dengue virus and at least one inactivated non-dengue flavivirus, and methods of making and using thereof. As used herein, such immunogenic compositions are referred to as "DENV+FV compositions".

[0021] A "flavivirus" refers to a virus species belonging to the genus *Flavivirus* in the family *Flaviviridae*. Flaviviruses can be grouped as set forth in the following Table 1:

Table 1
Tick-borne viruses
• Mammalian tick-borne virus group o *Absettarov virus* o *Alkhurma virus* (ALKV) o *Deer tick virus* (DT) o *Gadgets Gully virus* (GGYV) o *Kadam virus* (KADV) o *Karshi virus* o *Kyasanur Forest disease virus* (KFDV) o *Langat virus* (LGTV) o *Louping ill virus* (LIV) o *Mogiana tick virus* (MGTV) o *Ngoye virus* (NGOV)

- *Omsk hemorrhagic fever virus* (OHFV)
- *Powassan virus* (POWV)
- *Royal Farm virus* (RFV)
- *Sokuluk virus* (SOKV)
- *Tick-borne encephalitis virus* (TBEV)
- *Turkish sheep encephalitis virus* (TSE)

- Seabird tick-borne virus group
  - *Kama virus* (KAMV)
  - *Meaban virus* (MEAV)
  - *Saumarez Reef virus* (SREV)
  - *Tyuleniy virus* (TYUV)

Mosquito-borne viruses

- Without known vertebrate host
  - *Aedes flavivirus*
  - *Barkedji virus*
  - *Calbertado virus*
  - *Cell fusing agent virus*
  - *Chaoyang virus*
  - *Culex flavivirus*
  - *Culex theileri flavivirus*
  - *Culiseta flavivirus*
  - *Donggang virus*
  - *Hanko virus*
  - *Ilomantsi virus*
  - *Kamiti River virus*
  - *Lammi virus*
  - *Marisma mosquito virus*
  - *Nakiwogo virus*
  - *Nounané virus*
  - *Nhumirim virus*
  - *Nienokoue virus*
  - *Palm Creek virus* (PCV)
  - *Spanish Culex flavivirus*
  - *Spanish Ochlerotatus flavivirus*
  - *Quang Binh virus*
- Aroa virus group
  - *Aroa virus* (AROAV)
  - *Bussuquara virus* (BSQV)
  - *Iguape virus* (IGUV)
- Dengue virus group
  - *Dengue virus* (DENV)
  - *Kedougou virus* (KEDV)
- Japanese encephalitis virus group
  - *Bussuquara virus*
  - *Cacipacore virus* (CPCV)
  - *Koutango virus* (KOUV)
  - *Ilheus virus* (ILHV)
  - *Japanese encephalitis virus* (JEV)
  - *Murray Valley encephalitis virus* (MVEV)
    - *Alfuy virus*
  - *Rocio virus* (ROCV)
  - *St. Louis encephalitis virus* (SLEV)
  - *Usutu virus* (USUV)
  - *West Nile virus* (WNV)
  - *Yaounde virus* (YAOV)
- Kokobera virus group
  - *Kokobera virus* (KOKV)

○ *New Mapoon virus* (NMV) ○ *Stratford virus* (STRV) • Ntaya virus group ○ *Bagaza virus* (BAGV) ○ *Baiyangdian virus* (BYDV) ○ *Duck egg drop syndrome virus* (BYDV) ○ *Ilheus virus* (ILHV) ○ *Jiangsu virus* (JSV) ○ *Israel turkey meningoencephalomyelitis virus* (ITV) ○ *Ntaya virus* (NTAV) ○ *Tembusu virus* (TMUV) • Spondweni virus group ○ *Spondweni virus* (SPOV) ○ *Zika virus* (ZIKV) • Yellow fever virus group ○ *Banzi virus* (BANV) ○ *Bamaga virus* (BGV) ○ *Bouboui virus* (BOUV) ○ *Edge Hill virus* (EHV) ○ *Jugra virus* (JUGV) ○ *Saboya virus* (SABV) ○ *Sepik virus* (SEPV) ○ *Uganda S virus* (UGSV) ○ *Wesselsbron virus* (WESSV) ○ *Yellow fever virus* (YFV)
Viruses with no known arthropod vector
• Tamana bat virus (TABV) • Entebbe virus group ○ *Entebbe bat virus* (ENTV) ▪ *Sokoluk virus* ○ *Yokose virus* (YOKV) • Modoc virus group ○ *Apoi virus* (APOIV) ○ *Cowbone Ridge virus* (CRV) ○ *Jutiapa virus* (JUTV) ○ *Modoc virus* (MODV) ○ *Sal Vieja virus* (SVV) ○ *San Perlita virus* (SPV) • Rio Bravo virus group ○ *Bukalasa bat virus* (BBV) ○ *Carey Island virus* (CIV) ○ *Dakar bat virus* (DBV) ○ *Montana myotis leukoencephalitis virus* (MMLV) ○ *Phnom Penh bat virus* (PPBV) ○ *Rio Bravo virus* (RBV)
Non vertebrate viruses
• *Soybean cyst nematode virus 5*
Viruses known only from sequencing
• *Aedes flavivirus* • *Aedes cinereus flavivirus* • *Aedes vexans flavivirus* • *Culex theileri flavivirus*

[0022] A dengue virus (DENV) is a species of the genus Flavivirus. There are 4 serotypes of dengue virus. As used herein, a DENV of serotype 1 is referred to as "DENV-1", a

DENV of serotype 2 is referred to as "DENV-2", a DENV of serotype 3 is referred to as "DENV-3", and a DENV of serotype 4 is referred to as "DENV-4". As used herein, a "non-dengue flavivirus" refers to a flavivirus other than one belonging to the dengue virus group as set forth in Table 1. Vaccination with a tetravalent inactivated dengue virus vaccine composed of a mixture of inactivated DENV-1, inactivated DENV-2, inactivated DENV-3, and inactivated DENV-4 results in protective immunity against all 4 serotypes. See Fernandez, et al. (2015) Am J Trop Med Hyg 92(4):698-708, which is herein incorporated by reference in its entirety.

[0023] Prior to the present invention, however, it was unknown whether vaccination with two or more different species of flavivirus would result in immunologic interference since different species of other genuses, such as alphaviruses, result in immunologic interference, and in view of conflicting evidence of immunologic interference with the 17D vaccine with prior infections by flaviviruses such as JEV. See e.g., McClain, et al. (1998) J Infect Dis 177:634-641; Thomas, et al. (2011) Curr Opin Infect Dis 24:442-450; and VACCINES, 6th Ed. Plotkin, et al. Elsevier Health Sciences, 2012, pp. 931-932. In other words, prior to the present invention, it was unknown whether vaccinating a subject with a composition comprising an inactivated dengue virus and an inactivated non-dengue flavivirus would result in immunologic interference, thereby preventing or reducing the protective efficacy against the dengue virus and/or the non-dengue flavivirus as compared to the protective efficacy conferred by vaccinating with only a single species of flavivirus. As disclosed herein, it was found that DENV+FV compositions, i.e., comprising a mixture of at least one inactivated dengue virus and at least one inactivated non-dengue flavivirus, provides a protective immune response without immunologic interference when administered to subjects.

[0024] Therefore, the present invention provides immunogenic compositions comprising, consisting essentially of, or consisting of one or more inactivated dengue virus (DENV) and one or more inactivated non-dengue flavivirus (FV). In some embodiments, the immunogenic compositions of the present invention result in an immune response without immunologic interference between the one or more inactivated DENV and the one or more inactivated FV when administered to a subject. In some embodiments, the immunogenic compositions according to the present invention comprise, consist essentially of, or consist of at least one inactivated DENV and one inactivated FV. In some embodiments, the immunogenic compositions according to the present invention comprise, consist essentially of, or consist of at least one inactivated DENV and two inactivated FVs. As used herein, the use of "consisting essentially of" means that the given immunogenic

composition may comprise one or more immunogenic agents (e.g., antibodies or antigens) in addition to the at least one inactivated DENV and the at least one inactivated FV so long as the immunogenic agents do not change the immunogenic efficacy of the combination of the at least one inactivated DENV and the at least one inactivated FV against the given DENV and FV (without the given immunogenic agent). As used herein, the use of "consisting of" means that the given immunogenic composition explicitly excludes additional immunogenic agents with the exception of immunogens resulting from the preparation and purification of the one or more inactivated DENV and the one or more inactivated FV, pharmaceutically acceptable carriers, adjuvants, auxiliaries, binders, buffers, colorants, diluents, dispersants, excipients, fillers, gelling agents, plasticizers, preservatives, solubilizing agents, stabilizers, solvents, surfactants, suspending agents, thickening agents, wetting agents, and the like, which are known in the pharmaceutical arts. In some embodiments, the adjuvant is alum, an aluminum salt such as aluminum hydroxide, or tocopherol such as AS03B or AS01E (GlaxoSmithKline). As used herein, "pharmaceutically acceptable carrier" is used interchangeably with "physiologically acceptable carrier" and includes any material that is non-reactive in the subject, and when combined with an active ingredient, allows the ingredient to retain biological activity.

[0025] The one or more inactivated dengue viruses and/or the one or more inactivated non-dengue flaviviruses in the DENV+FV compositions according to the present invention may be purified. As used herein, a "purified" inactivated virus means that an amount of the macromolecular components of the cells used to propagate the virus have been removed. In some embodiments, the amount removed from the one or more inactivated viruses is at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% of the macromolecular components. In some embodiments, the DENV+FV compositions according to the present invention are free of at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% of the macromolecular components.

[0026] In some embodiments, the DENV+FV compositions according to the present invention may be administered to subjects to induce the production of antibodies against the dengue virus(es) and the non-dengue flavivirus(es) in the given DENV+FV composition. Administration of the DENV+FV compositions according to the present invention may be carried out by any suitable means, including parenteral injection (such as

intraperitoneal, subcutaneous, or intramuscular injection), using methods known in the art. A DENV+FV composition according to the present invention may be administered to a subject who is susceptible to or otherwise at risk of infection by or exposure to the dengue virus(es) and/or the non-dengue flavivirus(es) in the given DENV+FV composition in an immunogenically effective amount. As used herein, an "immunogenically effective amount" refers to an amount that induces an immune response, e.g., antibodies (humoral) against the dengue virus(es) and the non-dengue flavivirus(es) in the given DENV+FV composition, when administered to a subject. The immunogen(s) in the compositions may also induce cellular (non-humoral) immune responses. Such immunogenically effective amounts can be readily determined using methods known in the art and depend on the subject's state of health and weight, the mode of administration, the nature of the formulation, etc. The amount of each inactivated virus in DENV+FV compositions according to the present invention may be adjusted, e.g., increased or decreased, to result in a formulation comprising immunologically effective amounts of each. The amounts of each inactivated virus in a given DENV+FV composition may be the same or different.

[0027] Single or multiple administration of the DENV+FV compositions according to the present invention may be carried out. Multiple administration may be required to elicit sufficient levels of immunity. Levels of induced immunity can be monitored by measuring amount of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection.

[0028] The following examples are intended to illustrate but not to limit the invention.

[0029] *Tetravalent Purified Inactivated Dengue Vaccine*

[0030] A tetravalent purified inactivated dengue vaccine (TDENV-PIV) was developed at the Walter Reed Army Institute of Research (WRAIR) based on DENV-1 (WP-74), DENV-2 (S16803), DENV-3 (CH53489), and DENV-4 (TVP-360) virus strains, which were adapted to Vero cells suitable for vaccine manufacture to produce Master and Production seeds. These dengue virus strains are also identified by ATCC designators VR-2649, VR-2650, VR-2654, and VR-2651, respectively. See U.S. Patent No. 6,190,859, which is herein incorporated by reference in its entirety. Each DENV strain was used to make a monovalent purified inactivated vaccine using methods known in the art and then combined as a mixture to give the tetravalent purified inactivated dengue vaccine. See e.g., U.S. Patent No. 6,254,873; Putnak, et al. (1996) J Infect Dis 174:1176-84; and Putnak, et al. (1996) Am J Trop Med Hyg 55:504-10, which are herein incorporated by reference in their entirety. Other purified inactivated dengue compositions comprising, for example, one DENV serotype, all 4 DENV serotypes, or any

combination of 2-3 DENV serotypes may be prepared and used according to the present invention. As used herein, "DENV1-4" refers to a mixture of inactivated dengue viruses comprising all 4 serotypes.

[0031] *Purified Inactivated Yellow Fever Vaccine*

[0032] The yellow fever virus (YFV) is a flavivirus species that belongs to the yellow fever virus group as set forth in Table 1.

[0033] Two inactivated yellow fever virus vaccines are 17DD and XRX-001. The 17DD vaccine is based on the YFV 17DD strain cultivated in Vero cells, inactivated with β-propiolactone, and adsorbed to aluminum hydroxide. See Pereira, et al. (2015) Vaccine 33(35):4261-4268, which is herein incorporated by reference in its entirety. The XRX-001 vaccine is based on the YFV 17D strain cultivated in Vero cells, inactivated with beta-propiolactone (BPL), and formulated with aluminum hydroxide. See Monath, et al. (2010) Vaccine 28(22):3827-3840; and Monath, et al. (2011) N Engl J Med 364:1326-1333, which are herein incorporated by reference in their entirety.

[0034] *Purified Inactivated Japanese Encephalitis Virus Vaccine*

[0035] Japanese encephalitis virus (JEV) is closely related to the West Nile virus (WNV) and the St. Louis encephalitis virus, which all belong in the JEV sero-complex. See Tsai TR, Solomon T, D. V. Flaviviruses (yellow fever, dengue, dengue hemorrhagic fever, Japanese encephalitis, West Nile, St. Louis encephalitis, tick-borne encephalitis). In: Mandell GL, Bennett JE, R. D, eds. Principals and practice of infectious diseases. 6th ed. Philadelphia: Elsevier; 2004:1926-1950, which is herein incorporated by reference.

[0036] The IXIARO® vaccine (Intercell USA, Inc., a subsidiary of Valneva Austria GmbH) is based on the JE SA14-14-2 virus strain propagated in Vero cells, concentrated, purified, inactivated with formalin, and formulated with aluminum hydroxide.

[0037] *Production of Inactivated Virus Vaccines*

[0038] For production of each inactivated virus for incorporation into the DENV+FV compositions exemplified in the experiments herein, banked Vero cells certified for vaccine production were seeded into four to five 850 cm^2 roller bottles, fed with Eagle's Minimal Essential Medium (EMEM)/10% Fetal bovine serum (FBS), and incubated on a roller apparatus (1 rpm) at 35°C until 95-100% confluent. Confluent cultures were infected with virus in 10 ml of EMEM at a multiplicity of infection (moi) of approximately 0.01 plaque-forming units (pfu) per cell, incubated at 35°C, at 1 rpm, then re-fed with 200 ml of EMEM/2% FBS. At day 3 post-infection (only trace of cytopathic effect evident) the cultures were washed 3X with serum free EMEM, 200 ml per wash,

then re-fed with 200 ml serum free EMEM. Culture supernatant fluids were harvested on day 5, 7, 9, and 11 post-infection, followed by re-feeding of the cultures with serum-free EMEM.

[0039] The harvested supernatants were clarified by filtration through a 0.45 μm filter unit to remove any whole cells or cell debris and then stored at 4°C the collected harvests (4-5 L) were pooled and concentrated approximately 80- to 100-fold by tangential flow ultrafiltration using a Filtron brand Omega 100K Screen Channel membrane ultrafilter cassette. The virus concentrate was treated with 2 mg/ml of protamine sulfate, incubated at 4°C, 2 hours, and centrifuged at 13,000 rpm, 5 minutes, 4°C, in a TY30 rotor to precipitate residual nucleic acids and some contaminating cellular proteins. The pooled virus supernatant were applied to 15-60% (w/w) sucrose gradients and centrifuged at 17,000 rpm, 18 hours, 4°C, in a Beckman SW28 rotor. The visible viral bands were collected in one ml fractions from the bottom of the tubes using a mineral oil displacement fraction collector. The virus peak was located by testing 10 μl of each fraction in a virus hemagglutination (HA) assay with goose red blood cells, and the fraction with the peak HA titer and fractions with at least one quarter of the peak titer were pooled.

[0040] Protein assay (Biorad) was performed on the virus pool to determine the appropriate dilution factor prior to inactivation. Virus inactivation was carried out in the presence of 1:2000 (5 mM) formalin at 22°C for 10 days with filtration through a 0.22 μm filter and transfer to a fresh container at 48 hours. At day 10, sodium bisulfite was added in equimolar amounts to neutralize the residual formalin and the composition comprising the inactivated virus was filtered using a 0.22 μm filter and stored in bulk at 4°C protected from light. Alternatively, diafiltration was used to remove residual formalin. The bulk compositions were assayed for sterility, protein, and antigen content using established standard operating procedures. Residual infectivity was assayed by inoculation of 5% of the lot volume onto Vero cell cultures and incubation for 14 days, sufficient to amplify any remaining infectious virus, which can then be detected by plaque assay on Vero cells. Following inactivation, the bulk compositions were mixed with aluminum hydroxide (an adjuvant designed to improve immunogenic potency) or other adjuvant, as appropriate, at the time of formulation as a DENV+FV composition. The DENV+FV compositions were formulated to contain 2.5 micrograms (μg) of each virus antigen based on previous monkey studies with alum-adjuvanted, inactivated DENV and JEV vaccines. See e.g., Fernandez, et al. (2015) Am J Trop Med Hyg 92(4):698-708, which is herein incorporated by reference in its entirety.

[0041] Prior to formulation into a DENV+FV composition, each bulk composition may be formulated with a given adjuvant (which may be the same or different) and then adjuvanted bulk compositions may be used to formulate a DENV+FV composition. Alternatively, the bulk compositions may be mixed together first and then one or more adjuvants may be added to the mixture.

[0042] Attenuated and inactivated flavivirus vaccines, such as the 17DD, XRX-001, and IXIARO® vaccines, may be obtained and used to make DENV+FV compositions according to the present invention. If an attenuated flavivirus vaccine is used, it may be inactivated using methods known in the art.

[0043] *Vaccine Potency Testing*

[0044] Immunogenic potency testing was performed by administering the individual monovalent vaccines or polyvalent vaccine combinations (DENV1-4, and DENV+FV compositions) by intramuscular injection to 5-6 week-old, female, Swiss-ICR mice, group sizes of 10, in four- to five-fold serially graded doses from one nanogram to one microgram per 0.1 ml dose, as required to reach an endpoint. An immunogenicity control group (N=10) received saline instead of vaccine. The mice were boosted on day 28 and bled on day 42. Sera were tested by micro-neutralization (MN) assay to determine seroconversion rates and virus neutralizing antibody titers for each virus. The median immunizing dose (ID_{50}) was calculated by probit. Vaccine potency may be monitored by periodic testing throughout the study.

[0045] *Non-Human Primate Studies*

[0046] Compositions comprising purified inactivated dengue viruses of serotypes DENV-1, DENV-2, DENV-3, and DENV-4 (DENV1-4) combined with either purified inactivated YFV 17D (DENV1-4+YFV) or purified inactivated JEV SA14-14-2 (DENV1-4+JEV) were used to vaccinate rhesus macaques to exemplify the immunogenic efficacy of DENV+FV compositions according to the present invention. Rhesus macaques are accepted animal models for demonstrating the immunogenicity and protective efficacy of flavivirus vaccines. Twenty (20) healthy adult rhesus macaques, Indian strain, both males or females, at least 4 kg in weight, flavivirus naïve including negative for antibodies to DENV serotypes 1-4, JEV, YFV, West Nile (WNV), and St. Louis Encephalitis (SLEV) viruses were used for this study. The animals were randomly assigned to groups (N=5 animal per group), vaccinated, boosted, and sera collected during the course of the study tested for virus neutralizing antibodies against DENV serotypes 1-4, YFV, and JEV. The results are set forth in Table 2. As shown in Table 2, DENV+FV compositions according

to the present invention are immunogenic, elicit virus type specific neutralizing antibodies against DENV and against the given non-dengue flavivirus, which increased 10-fold or more after the second dose. Specifically, the DENV1-4+YFV and the DENV1-4+JEV compositions resulted in neutralizing antibodies against each virus present in the given composition with no evidence of immunologic interference among different viral antigens. In particular, the results show that compositions comprising inactivated dengue viruses and inactivated non-dengue flaviviruses (DENV+FV compositions) do not result in immunologic interference when administered to subjects.

Table 2

Immune Responses of Rhesus Macaques Vaccinated with a DENV+FV Composition

Vaccine Groups (N = 5)	Immunizations[1]	Group geometric mean virus neutralizing antibody titers vs.					
		JEV	YFV	DENV-1	DENV-2	DENV-3	DENV-4
DENV1-4 alone	Pre-vaccination	<10	<10	<10	<10	<10	<10
	Post dose-1	<10	<10	83	121	114	55
	Post dose-2	<10	<10	712	2255	1610	2295
DENV1-4+YFV[3]	Pre-vaccination	<10	<10	<10	<10	<10	<10
	Post dose-1	<10	252	71	89	44	42
	Post dose-2	<10	1507	735	968	1318	1309
DENV1-4+JEV[4]	Pre-vaccination	<10	<10	<10	<10	<10	<10
	Post dose-1	20	<10	29	38	61	49
	Post dose-2	771	<10	473	1097	882	934
Placebo[5]	Pre-vaccination	<10	<10	<10	<10	<10	<10
	Post dose-1	<10	<10	<10	<10	<10	<10
	Post dose-2	<10	<10	<10	<10	<10	<10

[1] Animals (5 per group) were immunized intramuscularly on days 0 and 28 with 0.5 ml of the indicated vaccine or with a placebo (negative control). Each vaccine contained 2.5 µg of each indicated virus type adsorbed to 0.5% aluminum hydroxide. Blood samples collected on days 0, 28, and 60 were tested for virus neutralizing antibodies by Micro-neutralization (MN) ELISA, with a cut-off of 1:10 (antibody negatives are <10).

[2] DENV1-4 = Composition comprising purified inactivated dengue viruses of serotypes 1 to 4 (DENV-1, DENV-2, DENV-3, and DENV-4) in a pharmaceutically acceptable carrier.

[3] DENV1-4+YFV = Composition comprising purified inactivated dengue viruses of serotypes 1 to 4 (DENV-1, DENV-2, DENV-3, and DENV-4) and purified inactivated YFV 17D in a pharmaceutically acceptable carrier.

[4] DENV1-4+JEV = Composition comprising purified inactivated dengue viruses of serotypes 1 to 4 (DENV-1, DENV-2, DENV-3, and DENV-4) and purified inactivated JEV SA14-14-2 in a pharmaceutically acceptable carrier.

[5] Phosphate-buffered saline (PBS) control

[0047] *Additional Non-Human Primate Vaccine Studies*

[0048] Additional non-human primate vaccine studies using DENV+FV compositions containing one or more inactivated DENV (including any combination of 1-4 DENV

serotypes) combined with one or more inactivated non-dengue flaviviruses selected from the Tick-borne viruses and Mosquito-borne viruses, such as YFV, JEV, WNV, SLEV, ZIKV, and combinations such as YFV+JEV, may be conducted as follows.

[0049] Healthy adult rhesus macaques, Chinese or Indian strain, male or female, at least 4 kg in weight, flavivirus naïve to DENV (serotypes 1-4) and also to the given inactivated non-dengue flavivirus and viruses closely related to the given inactivated non-dengue flavivirus (e.g., if the inactivated non-dengue flavivirus is JEV, then the macaques should also be naïve to WNV and SLEV) as demonstrated by negative PRNT and/or HAI assays. Preferably, the macaques are also simian retrovirus (STLV, SIV) negative.

[0050] The animals are randomly allocated (e.g., by block randomization using nQuery Advisor) to receive the test compositions, i.e., the given DENV+FV composition and controls (e.g., vehicle, a composition comprising only the inactivated DENV present in the given DENV+FV composition, and/or a composition comprising only the inactivated non-dengue flavivirus(es) present in the given DENV+FV composition. Prior to all out-of-cage procedures the animals may be anesthetized with Ketamine/Acepromazine administered intramuscularly. The test compositions are administered intramuscularly in a suitable large muscle (e.g., deltoid) according to a given schedule such as that provided in Table 3.

[0051] Following vaccinations, the animals are observed for local side effects (e.g., redness, swelling) and systemic side effects (e.g., lethargy, loss of appetite). Conventional IgM and IgG ELISA performed on sera collected 7 to 14 days after immunization may be used to discriminate between primary and secondary-type immune responses in the animals. Virus neutralizing antibodies in sera is measured by virus plaque reduction neutralization test (PRNT) or microneutralization (MN) assay for DENV (all 4 serotypes) and the given non-dengue flavivirus(es) at selected time points. Neutralizing antibodies directed against serotype-specific epitopes in domain 3 of the virus envelope (E) protein (EDIII) may be measured by a competitive Luminex® assay (described below) for the four DENVs, and also for the given non-dengue flavivirus(es). A competitive antibody-capture ELISA may be performed using antigens prepared from virus infected cell culture supernatants. At a given time after vaccination, the animals are challenged with live dengue virus and/or a live form of the given non-dengue flavivirus(es) by subcutaneous inoculation. Blood is collected at given intervals for measuring the animals' immune responses to vaccination and viremia (as an infection marker) after virus challenge. If the non-dengue flavivirus is JEV, since there is no BSL-2 JE challenge virus strain available

that is capable of inducing viremia, anti-JEV neutralizing antibody titers of 1:10 or greater following vaccination may be used as a protection correlate.

Table 3		
Study Day	**Event**	**Blood Collection Volume (total)**
-14	Pre-study screening (PRNT, HAI)	5 ml (5 ml)
0	Study baseline (for antibody assays) Vaccine dose 1	5 ml (10 ml)
14	Antibody measurements	5 ml (15 ml)
28	Antibody measurements Vaccine dose 2	5 ml (20 ml)
35	Antibody measurements	5 ml (25 ml)
42	Antibody measurements	5 ml (30 ml)
60	Antibody measurements (groups 1, 3, and 4 go off-protocol)	5 ml (35 ml)
150	Antibody measurement. Virus Challenge	5 ml (40 ml)
151-164	Viremic phase blood collections	2 ml per animal per day for 14 consecutive days (68 ml)
180	Final bleed for antibody to virus challenge	5 ml (73 ml)

[0052] A. Production of vaccine and control formulations: Vero cells qualified for vaccine production are seeded into four to five 850 cm^2 roller bottles, fed with EMEM/10% FBS, and incubated on a roller apparatus (1 rpm) at 35°C until 95-100% confluent. Confluent cultures are infected with virus in 10 ml of EMEM at a multiplicity of infection (moi) of approximately 0.01 plaque-forming units (pfu) per cell, incubated at 35°C, at 1 rpm, then re-fed with 200 ml of EMEM/2% FBS. At day 3 post-infection (or when there is just a trace of cytopathic effect evident) the cultures are washed 3X with serum free EMEM, 200 ml per wash, then re-fed with 200 ml serum free EMEM. Culture supernatant fluids are harvested on day 5, 7, 9, and 11 post-infection, followed by re-feeding of the cultures with serum free EMEM. The harvested supernatants are clarified by filtration through a 0.45 μm filter unit to remove any whole cells or cell debris and then stored at 4°C. The collected harvests (4-5 L) are pooled and concentrated approximately 80- to 100-fold by tangential flow ultrafiltration using a Filtron brand Omega 100K Screen Channel membrane ultrafilter cassette. The virus concentrate are treated with 2 mg/ml of protamine sulfate, incubated at 4°C, 2 hours, and centrifuged at 13,000 rpm, 5 minutes, 4°C, in a TY30 rotor to precipitate residual nucleic acids and some contaminating cellular proteins. The pooled virus supernatant will then be applied to 15-60% (w/w) sucrose gradients and centrifuged at 17,000 rpm, 18 hours, 4°C, in an SW28 rotor. The visible

viral bands are collected in one ml fractions from the bottom of the tubes using a mineral oil displacement fraction collector. The virus peak are located by testing 10 µl of each fraction in an HA assay with goose red blood cells, and the fraction with the peak HA titer and fractions at least one quarter of the peak titer are pooled. Protein assay (Biorad micro BCA) are performed on the virus pool to determine the appropriate dilution factor prior to inactivation. Virus inactivation are carried in the presence of a 1:2000 dilution of formalin (5 mM) at 22°C for 10 days with filtration through a 0.22 µm filter and transfer to a fresh container at 48 hours. At day 10, the compositions are diafiltered to remove residual formalin and then sterile filtered through a 0.22 µm filter and stored in bulk at 4°C protected from light. The bulk compositions are assayed for sterility, protein content, and antigen content using established SOPs. Residual viral infectivity are tested by inoculation of 5% of the vaccine lot volume onto Vero cell cultures and incubation for 14 days, sufficient to amplify any remaining infectious virus, which will then detected by plaque assay on Vero cells.

[0053] Prior to testing in rhesus macaques, the vaccines are tested for immunogenicity in mice, e.g., at Harlan Biosciences (Harlan Protocol 421-09. Rodent Immunization for Antibody Production. IACUC approval November 2012), and then formulated to a desired dosage with a given adjuvant such as aluminum hydroxide (0.1 to 0.5%). For example, potency testing may be performed by administering the vaccines by IM injection to 5-6 week-old, female, Swiss-ICR mice, in four- to five-fold serially graded doses from one nanogram to one microgram per 0.1 ml dose, as required to reach a given endpoint. An immunogenicity control group is administered saline instead of vaccine. The mice are boosted on day 28, exsanguinated on day 42, and the blood processed to sera. The sera is aliquoted and frozen at -20°C. The mouse sera is assayed by PRNT to determine seroconversion rates (50% or greater plaque reduction at the 1:10 assay cut-off) and virus neutralizing antibody titers for each virus. The median (50%) immunizing dose (ID_{50}) will be calculated by probit analysis (SPSS). Vaccine potency may be monitored by periodic testing throughout the study.

[0054] B. Viruses. Viruses used in the serological assays may be low-passage level, e.g., Vero cell adapted stocks of DENV-1 (WP 74), DENV-2 (S16803), DENV-3 (CH53489), DENV-4 (341750 or TVP-360), inactivated JEV SA14-14-2, attenuated YFV 17D inactivated YFV 17D, and an attenuated WN replicon (for laboratory PRNT assays).

[0055] C. Plaque reduction neutralization test (PRNT). Test sera are diluted in EMEM with 2% heat-inactivated FBS serially, four-fold, from 1:10 to 1:640 or greater depending on the titer. An equal volume of each flavivirus present in the given DENV+FV

composition adjusted to an infectivity concentration of 500 PFU/ml are added to each serum serial dilution tube and incubated at 35°C for 30 minutes. After incubation, 0.2 ml of each serum-virus mixture are inoculated to six-well plates (in triplicate wells) containing confluent monolayers of Vero-81 cells (ATCC). The plates are rocked gently and incubated at 35°C with 5% CO_2 for 60 minutes to allow for virus adsorption, then the cell monolayers are overlayered with 2.5 ml of a 1% nutrient agarose overlay (EMEM, 2% FCS), L-Glut, NEAA, 1% LMA Sea Plaque agarose) and incubated at 35°C with 5% CO_2 for 3-5 days depending on the virus plaque size. Viral plaques are visualized by staining with neutral red (Sigma) in 1% agarose/saline and incubated at 35°C with 5% CO_2 for 12-24 hours. Plaques are counted and plaque reduction 50% endpoint ($PRNT_{50}$) titers are calculated by probit analysis implemented on a PC running SPSS statistical software (SPSS Inc.).

[0056] D. Microneutralization (MN) ELISA. Serum samples are serially diluted three-fold in 96-well micro-plates for dilutions from 1:10 to 1:7,290 (if needed). One hundred microliters of a calibrated virus dose (the highest virus dilution that produces $\geq$ 1.0 ELISA OD_{405} value after a five-day incubation) are added to 100 µL of each serum dilution and incubated at room temperature for 2 hours. After incubation, the contents of the plates are transferred to microplates containing confluent Vero WHO cell monolayers from which the growth medium has been decanted. Positive (virus-only) and negative (growth medium alone) controls are included in each assay. After incubation for 4-5 days, the cells are fixed with absolute ethanol:methanol (1:1) for 1 hour at -20°C, washed three times with PBS. After washing, 100 µL of 1:16,000 dilution of anti-DEN mAb 4G2 (CDC) in antibody sample diluent are added to each well and incubated at 35°C for 2 hours, then wash 5 times with PBS. A detection antibody (HRP-conjugate) diluted 1:4,000 are added to the plates (100 µL/well) and incubated at 35°C for 1 hour. After incubation, the plates are washed 5 times with PBS and 100 µL/well of HRP substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Kirkegaard and Perry Laboratories) are added to each well. The plates are read (OD_{405}) on a microplate reader (Titertek-Ascent). For a valid assay, the average OD_{405} of three non-infected control wells of each microplate must be $\leq$ 0.4; this value are used for normalization. The virus-only control wells must have an $OD_{405} \geq 1.0$. The normalized OD values are transformed to a four-parameter logistical model using Graph Pad Prism version 4.0 software for Windows (Graph Pad Software) to calculate 50% MN titers using a sigmoidal dose response (variable slope) formula. The virus neutralization titer is the reciprocal of the serum dilution giving 50% reduction in the absorbance readout of the assay when compared with the virus dose control without

serum. For a valid 50% MN titer, the R^2 value for the fitted sigmoidal curve must be ≥ 0.80

[0057] E. Competitive Multiplexed Luminex Immunoassay. Recombinant E domain III (EDIII) proteins of each flavivirus present in the given DENV+FV composition are covalently coupled to free carboxyl groups on xMAP Multi-Analyte COOH Microspheres (spectrally discrete polystyrene beads from Luminex Corporation) using an N-hydroxysulfosuccinimide (sulfo-NHS)-enhanced carbodiimide-mediated conjugation reaction. Monoclonal Abs are directly conjugated to R-phycoerythrin (PE) via succinyl esters containing either a thiol or maleimide reactive group. Microspheres coupled to each recombinant EDIII protein are diluted to a final concentration of 8.0×10^5 microspheres/ml and 25 µl are added to each well of a black, opaque microtiter plate (Costar). A standard reference serum pool (containing anti-flavivirus neutralizing antibodies against flavivirus present in the given DENV+FV composition) and single reference sera are used to generate a standard curve. An antibody negative serum control may also be included in each assay. Following incubation the PE-tagged type-specific mAbs (mAb-PE) are added to each well of the plate in a volume of 25 µl and mixed. After incubation, all samples are transferred to a filter plate (Millipore) pre-wetted with PBS. The serum samples are washed three times with 200 µl of PBS buffer and the EDIII-microspheres are resuspended in 200 µl of PBS plus 1% BSA for analysis on a Luminex® 200™ Total System and IS 2.3 software designed for template-based data acquisition with data regression analysis. The antibody titers of test sera are obtained by measuring the ability of a test sample to inhibit mAb-PE binding to the recombinant EDIII proteins and comparing that value to that of a dilution series of the reference standard. Specifically, relative inhibition of mAb-PE binding are compared to a standard curve by using a five-parameter logistic (5PL) function in curve fitting software, and the test sample concentration are calibrated from the fitted standard curve. The standard reference sera used for the standard curve are assigned arbitrary antibody concentrations. Data, in median fluorescent intensity (MFI) units, are processed in Bio-Plex Manager 3.0 software (Bio-Rad) or using Microsoft Excel spreadsheet (Microsoft).

[0058] F. Indirect competitive Ab-Capture ELISA. Recombinant EDIII proteins are used to coat microtiter plates. Test sera and pre-titrated type-specific neutralizing mAbs labeled with biotin are added to the pre-coated wells. After an appropriate incubation, streptavidin conjugated with enzyme (e.g., HRP), are added to each well followed by an enzyme substrate for color development. The results are read on a microplate reader (Titertek-

Ascent), and antibody titers determined by a comparison with a standard curve generated from serial dilutions of appropriate serum standards

[0059] G. Viremia assay. Vero cells in 25 cm^2 (T25) flasks are infected with 0.1 ml of serum and incubated at 35°C for 14 days (with a complete medium change at day 7) to amplify any virus that might be present. Virus are detected by plaque assay on Vero cell monolayers stained with neutral red. Direct plaque assay of virus positive sera will then be performed to obtain viremia titers (pfu/ml). Viremia are measured independently by PCR assay.

[0060] H. Specimen handling. Blood samples are processed to sera by centrifugation at 2,500 x *g*, 10 minutes, 20°C, and sera are divided into appropriately labeled aliquot tubes and stored frozen at -80°C prior to assay.

[0061] However, p-values presented for these analyses are considered to represent descriptive measures of strength of evidence rather than formal statistical inference. Fisher's exact test are used to compare protection rate and sero-conversion rates between control and vaccine groups. Repeated measure ANOVA are used to compare antibody titers between experimental groups. All titers are log transformed to stabilize variance. Kaplan Meier Curve are used to graph the time to viremia, and are compared between experimental groups using log-rank test.

[0062] I. Vaccination. Animals are inoculated with vaccines or controls IM with a 23 to 25 gauge, 5/8-in. to 1-in. needle and a 1 cc syringe in the upper arm. Vaccinations and virus challenge are carried out using aseptic technique after first shaving and cleaning and disinfecting the site with 70% isopropyl alcohol. The volume administered are 0.5 ml. Booster vaccinations are administered in alternate arms, but otherwise identically to the primary vaccination and according to a given schedule, e.g., Table 3.

[0063] J. Virus challenge. Challenge virus doses of 4 to 5 log10 plaque-forming units (pfu) per ml are administered by subcutaneous (SC) inoculation of 0.5 ml of virus in sterile culture medium. The inoculation site are the loose skin of the upper medial aspect of the arm with a 23 to 25 gauge, 5/8-in. to 1-in. needle and a 1 cc syringe. Prior to inoculation, the site are shaved cleaned and disinfected with 70% isopropyl alcohol. Residual challenge virus are returned to the laboratory to verify its potency by plaque titration. Daily after challenge, for a period of 14 days, 2 ml of blood are drawn for determining the titers of circulating virus (viremia). Rhesus monkeys do not show any signs of disease following subcutaneous virus challenge; measurement of viremia, which is thought to correlate with human disease, will allow for assessment of the protective efficacy of the vaccine.

[0064] Rhesus macaques were immunized with a DENV1-4+YFV composition and later challenged with DENV-2 or YFV as set forth above. The results in Table 4 evidence that DENV+FV compositions according to the present invention provides protective immunity against the dengue virus(es) and the non-dengue flaviviruses virus(es) in the given DENV+FV.

Table 4
Responses to Vaccination[1] and Virus Challenge in Rhesus Macaques Receiving DENV1-4+YFV

Vaccine formulation	Neutralizing antibody titers on day of challenge: Mean (range)		Challenge virus	Viremia positive animals/Total animals	Days of viremia
DENV1-4+YFV[2]	JEV	<10	Dengue type-2	0/5	0
	YFV	893 (161-1466)			
	DENV-1	145 (67-263)			
	DENV-2	105 (43-192)			
	DENV-3	80 (51-174)			
	DENV-4	273 (86-730)			
	JEV	<10	Yellow fever	0/5	0
	YFV	721 (194-2295)			
	DENV-1	347 (198-621)			
	DENV-2	310 (163-693)			
	DENV-3	209 (53-304)			
	DENV-4	410 (234-534)			
Placebo[3]	N/A	N/A	Dengue type-2	5/5	10
	N/A	N/A	Yellow fever	1/5	1

[1] Animals (5 per group) were immunized intramuscularly on days 0 and 28 with 0.5 ml of DENV1-4+YFV (contained 2.5 µg of each virus type adsorbed to 0.5% aluminum hydroxide) or with a placebo (negative control). Blood samples collected on days 0, 28, and 60 were tested for virus neutralizing antibodies and viremia.

[2] DENV1-4+YFV = Composition comprising purified inactivated dengue viruses of serotypes 1 to 4 (DENV-1, DENV-2, DENV-3, and DENV-4) and purified inactivated YFV 17D in a pharmaceutically acceptable carrier.

[3] Phosphate-buffered saline (PBS) control.

Therefore, in some embodiments, the present invention is directed to methods for immunizing subjects against one or more inactivated DENV and one or more inactivated non-dengue flaviviruses by administering to the subject a DENV+FV composition.

[0065] *DENV+FV Composition Studies in Humans*

[0066] Healthy male and female volunteers, ages 18-45, are examined and screened by a panel of tests, including blood chemistries, hematology, prothrombin time, partial thromboplastin time, urinalysis, rapid plasma reagin antibody, and serology for hepatitis B surface antigen and antibody to HIV. Volunteers are excluded on the basis of persistent significant abnormality or positive test. Volunteers are also excluded if they had previous

flavivirus immunity, which may affect responses to dengue vaccines or a history of allergy to neomycin, streptomycin, or gentamycin. Prior flavivirus immunity is defined as having no detectable hemagglutination inhibition antibodies (at a 1:10 serum dilution) against DENV (all 4 serotypes), JEV, or YFV, and no history of being vaccinated with a flavivirus or a prior flavivirus infection.

[0067] A standard randomized, single-blind inpatient clinical protocol is used for all pilot studies. Each volunteer is vaccinated with a given dose of the given DENV+FV composition. Volunteers are monitored closely for three weeks for adverse symptoms. Blood may be collected from volunteers according to a given schedule, e.g., every other day and on day 31, for routinely available medical tests for hemoglobin and hematocrit, white blood cell count with differential count, platelet count, and aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels, and antibody studies. Antibody tests may include ELISA, HAI, and plaque reduction neutralization tests (PRNT) performed using the same strains of flaviviruses provided in the given DENV+FV composition. Detection of anti-flavivirus IgM antibodies is performed by modification of an ELISA, where values >0.10 OD units are considered positive as previously described in the art. The HAI test is performed by the standard technique modified to microvolumes using 4-8 units of individual antigens, using serum extracted with acetone to remove inhibitors. See Clarke and Casals (1958) Am J Trop Med Hyg 7:561–573. PRNT assays are performed using methods known in the art.

[0068] A vaccine failure is defined as an unacceptable adverse clinical response or failure to develop convalescent IgM or PRNT antibodies.

[0069] Section headings are used for organizational purposes only and are not to be construed as defining or limiting the subject matter described. Unless explicitly provided otherwise, singular word forms include the plural forms. As used herein, “at least one” and “one or more” are used interchangeably. As used herein, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. As used herein, “and/or” means “and” or “or”. For example, “A and/or B” means “A, B, or both A and B” and “A, B, and/or C” means “A, B, C, or a combination thereof” and said “combination thereof” means “A and B, A and C, or B and C”.

[0070] To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so incorporated.

[0071] Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

WHAT IS CLAIMED IS:

1. An immunogenic composition comprising one or more inactivated dengue viruses and one or more inactivated non-dengue flaviviruses and a pharmaceutically acceptable vehicle.

2. The immunogenic composition of claim 1, wherein the one or more inactivated dengue viruses are selected from the group consisting of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4.

3. The immunogenic composition of claim 1, wherein the one or more inactivated dengue viruses consist of an inactivated DENV-1, an inactivated DENV-2, an inactivated DENV-3, and an inactivated DENV-4.

4. The immunogenic composition according to any one of claims 1 to 3, wherein said dengue virus is an inactivated DENV-1 produced from deposited DENV-1 strain having ATCC accession number VR-2649.

5. The immunogenic composition according to any one of claims 1 to 3, wherein said dengue virus is an inactivated DENV-2 produced from deposited DENV-2 strain having ATCC accession number VR-2650.

6. The immunogenic composition according to any one of claims 1 to 3, wherein said dengue virus is an inactivated DENV-3 produced from deposited DENV-3 strain having ATCC accession number VR-2654.

7. The immunogenic composition according to any one of claims 1 to 3, wherein said dengue virus is an inactivated DENV-4 produced from deposited DENV-4 strain having ATCC accession number VR-2651.

8. The immunogenic composition according to any one of claims 1 to 7, wherein the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever group viruses and Japanese encephalitis group viruses.

9. The immunogenic composition according to any one of claims 1 to 7, wherein the one or more inactivated non-dengue flaviviruses are selected from the group consisting of yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV).

10. The immunogenic composition according to any one of claims 1 to 7, wherein the one or more inactivated non-dengue flaviviruses are yellow fever virus (YFV) and Japanese encephalitis virus (JEV).

11. The immunogenic composition according to any one of claims 1 to 7, wherein the one or more inactivated non-dengue flavivirus is Zika virus (ZIKV).

12. The immunogenic composition according to any one of claims 1 to 11, wherein the one or more inactivated dengue viruses are present in an immunologically effective amount and/or the one or more inactivated non-dengue flaviviruses are present in an immunologically effective amount.

13. The immunogenic composition according to any one of claims 1 to 12, wherein the one or more inactivated dengue viruses are purified and/or the one or more inactivated non-dengue flaviviruses are purified.

14. The immunogenic composition according to any one of claims 1 to 13, and further comprising an adjuvant.

15. A method of producing antibodies against a dengue virus and a non-dengue flavivirus in a subject, which comprises administering to the subject the immunogenic composition according to any one of claims 1 to 14.

16. The method according to claim 15, wherein the immunogenic composition is administered as a single dose on a 0-28 day vaccination schedule.

17. The method according to claim 15 or claim 16, wherein the administration is by intramuscular injection.

18. A medicament for producing antibodies against a dengue virus and a non-dengue flavivirus in a subject, which comprises the immunogenic composition according to any one of claims 1 to 14.

19. Use of the immunogenic composition according to any one of claims 1 to 14.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/21696

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/12, A61K 39/395 (2016.01)
CPC - A61K 39/12, C12N 7/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (8): A61K 39/12, A61K 39/395 (2016.01)
CPC: A61K 39/12, C12N 7/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/204.1; 424/130.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Google patents, Google scholar, Google web, PatBase, Proquest Dialog
Combine/together; inactivated; vaccine/immunogen; flavivirus; Dengue virus/DENV/DENV-1/DENV-2/DENV-3/DENV-4; non-dengue flavivirus; yellow fever virus/YFV/Japanese encephalitis virus/JEV/West Nile virus/WNV/Zika virus/ZIKV

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IMOTO et al. "Dengue tetravalent DNA vaccine increases its immunogenicity in mice when mixed with a dengue type 2 subunit vaccine or an inactivated Japanese encephalitis vaccine" Vaccine. 22 January 2007. Vol. 25. No. 6. pp 1076-84. especially, Abstract; page 1082, Col. 1, para 3	1-7
Y	US 6,254,873 B1 (PUTNAK et al.) 3 July 2001 (03.07.2001) Abstract; claim 2; claim 5; claim 8; claim 11; claim 12; claim 13; claim 14; claim 15; claim 17; claim 32; Col. 3, ln 3-14	1-7
A	US 2013/0295162 A1 (SCHLESINGER et al.) 7 November 2013 (07.11.2013) Abstract; claim 38; para [0008]; para [0014]; para [0170]	1-7

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 2016 (12.05.2016)	02 JUN 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/21696

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,

[Continued on next page]

(54) Title: VACCINE COMPOSITIONS

(57) Abstract: The present disclosure provides vaccine compositions for prophylaxis and treatment of Zika virus infections comprising Zika virus antigens in immunogenic compositions, and in combination of Zika antigens with one or more arbovirus antigens such as Chikungunya virus and Japanese encephalitis virus antigens, methods of preparation and production of such compositions for use as vaccines for eliciting immune response in mammals against the above mentioned pathogens.

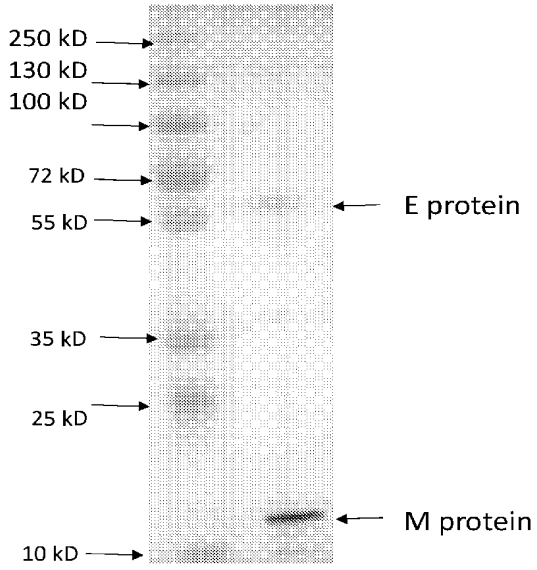


Figure 1

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SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

VACCINE COMPOSITIONS

FIELD OF THE INVENTION

The present invention discloses vaccine compositions comprising Zika virus antigens for prophylaxis and treatment of Zika virus infections in mammals. The invention also discloses stable vaccine compositions comprising Zika virus antigens with one or more arbovirus antigens such as Chikungunya and/ or Japanese encephalitis virus antigens. The present invention also relates to the methods of preparation, formulation and use of the same for simultaneously eliciting immune response to each of the above mentioned pathogens in mammals, and suitable for immunizing human subjects

BACKGROUND OF THE INVENTION

At present there is no vaccine available in the world for prophylaxis or treatment against Zika virus infections. Therefore, there is no prior art relevant to the invention disclosed in this application. However, for general understanding of the background and objectives behind this invention is discussed hereinafter in below paragraphs.

The Inventors of this patent application anticipated the epidemic potential of Zika virus in regions with high prevalence of *Aedes* mosquitoes, particularly *Aedes aegypti* that transmits the virus. The interest in initiating the Zika vaccine project early on, several months before the causal link of Zika virus infection to Guillain Barre Syndrome and to microcephaly became public knowledge in December 2015, was that there was no preparedness in any country in the world, nor measures initiated by anyone at that time to develop a vaccine to stop the ongoing virus transmission in countries such as Brazil, and to prevent further transmission in countries at risk for Zika virus. Increased International travel to and from regions with ongoing virus transmission impose a major risk to initiate an outbreak in countries with high prevalence of *Aedes* mosquitoes, particularly *Ae.aegypti* and those having a large naïve population hither to unexposed to the virus. The clinical picture of Zika virus infection in the early stages with characteristic high fever, maculopapular rashes and arthralgia is strikingly similar to the early onset symptoms of Chikungunya and Dengue virus infections that make differential diagnosis particularly challenging.

Zika virus vaccine project was initiated at the time when very little or no information was available on virus pathogenesis, genetic diversity, transmission, diagnosis, serological correlates for protection or animal models to test the vaccine concepts. From vaccine point of view, there was no information on whether the virus can be cultured *in vitro* in cell substrates and if yes, which cell substrates are best suitable, mechanism of adaptation to cells, potential virus titers and the feasibility to manufacture the vaccine product for human administration, as the published information at that time pertained to passaging the virus in mouse brain which is not suitable for vaccine production. Bharat Biotech initiated steps to start Zika vaccine project in late 2014, and commenced the experimental work soon thereafter resulting into this said patent application.

Arbovirus (arthropod-borne) infections are caused by viruses that are spread by arthropods such as mosquitoes. They cause significant human illness ranging from mild, asymptomatic infection to acute encephalitis or hemorrhagic fever that can prove fatal. The most significant

arboviruses causing human illness belong to three viral families, *Togaviridae*, *Flaviviridae*, and *Bunyaviridae*. Arbovirus infections are rampant in developing countries and cause severe morbidity particularly in the elderly population. The common characteristic feature of arbovirus infections caused by Dengue, Chikungunya, Zika, Japanese encephalitis and West Nile viruses among others is fever, headache, myalgia, joint pains with swelling and maculopapular rashes during the acute phase of the viral infection. Arthralgia is particularly a characteristic feature of Chikungunya, Dengue and Zika virus fever. Co-infections are common as the arboviruses largely share the same mosquito vectors such as for example Dengue, Chikungunya and Zika viruses that are transmitted by *Aedes* mosquitoes. Japanese encephalitis virus and West Nile viruses are transmitted predominantly by *Culex* mosquitoes. The problem is acute in developing countries where mosquito vector control programs have been ineffective and largely unsuccessful. The problem is compounded by the fact that there are no robust diagnostic methods available for diagnosing the disease causing viruses with certainty. International travel has aided widespread dissemination of these infectious agents, and diseases like Dengue and Chikungunya hitherto confined to tropical countries are now spread geographically to new areas and to temperate regions. Zika virus is reportedly spread to over 65 countries in the last two years. Autochthonous epidemic outbreaks reported in few countries in these regions are sustained by the local population of mosquito vectors.

Zika virus (ZIKV) is an emerging zoonotic arbovirus, belonging to the *Flaviviridae* family. Like Dengue and Chikungunya viruses, Zika virus can also be transmitted by *Aedes* mosquitoes more specifically *A. furcifer*, *A. taylori*, *A. luteocephalus*, *A. africanus*, *A.albopictus* and predominantly by *A.aegypti.* Travel tourism to nations where the recent epidemics were reported such as Polynesia has aided the geographical spread of the virus infection to Brazil, Columbia, Italy and to other countries. An autochthonous outbreak of the virus was reported in Italy caused by thelocally established *Aedes* mosquitoes. In Asia, Zika virus infection has occurred sporadically in Cambodia, Thailand, Indonesia, Malaysia and Bangladesh although large epidemic outbreaks have not been reported in these regions.

Chikungunya virus (CHIKV) is an *Alphavirus* of the family *Togaviridae*. The virus causes self-limiting febrile infection characterized by acute onset of high fever, headache, myalgia, arthralgia, swelling in joints and maculopapular rashes. Severe symptoms such hemorrhagia, fulminant hepatitis and neurological symptoms were reported in the more recent epidemics. Chikungunya virus is transmitted by both the *Aedesaegypti* and *Aedes albopictus* mosquitoes. Japanese encephalitis virus (JEV) is also a flavivirus of the family *Flaviviridae* and is transmitted largely by the *Culex* mosquitoes. JEV is related to Dengue, Yellow fever virus, Zika and West Nile viruses. JEV infection is largely asymptomatic, but in general it causes malaise with fever, headache and other flu-like symptoms. Rarely, the clinical infection progresses to encephalitis with seizures, spastic paralysis, coma and death. Children are particularly susceptible. In the countries endemic for JEV, most adults have natural immunity after childhood infection. Adults not exposed to the infection during childhood are susceptible at any age. The case-fatality rate in JEV caused by encephalitis can be as high as 30%. Neurological complications or psychiatric sequelae occur in high proportion of the cases with encephalitis. Globally, about 3 billion population is at risk for JEV infection. A few vaccines for prophylaxis of JEV infection have been successfully commercialized. Dengue virus (DENV) is a member of *Flaviviridae* family. The arbovirus infections can no longer be considered region specific as they are now geographically widespread and are significant public health problem in many parts of the world. The morbidity caused by the aforementioned arbovirus infections is usually high, and arthralgia in particular, adversely impacts physical mobility of the patients. Zika virus causes more serious congenital birth

deformities during infection in pregnancy, and Zika related Guillain Barre syndrome have been confirmed in the ongoing epidemics. Like any other viral infections, no specific therapeutics is available. Prophylactic vaccination can effectively interrupt Zika virus transmission and a vaccine would be the front line of defense from the Zika virus disease.

With this in mind, an effective strategy was developed to prevent further transmission of Zika virus to protect naïve population in countries with ongoing epdiemics and in countries where active Zika virus transmission has not been reported as yet. A combination vaccine for arbovirus infections is good strategy to protect vulnerable population from debilitating illnesses caused by Dengue, Chikungunya, Zika, Japanese encephalitis, West Nile and Yellow Fever viruses. Vaccines for JEV, Yellow Fever and one for Dengue serotypes has been commercialized and those for West Nile and CHIKV are in clinical development. There is no vaccine for Zika virus infection as yet, and the current invention discloses the methods for development of the first candidate Zika vaccines.

However, the choice of antigens to include in such a vaccine kit depends on several factors. The antibody dependent enhancement of virus caused by the Dengue serotypes is well researched and published and so also the cross reactivity of flavivirus antibodies. But what was not clear is that if there would be such interference or cross reactivity of prevalent Chikungunya antibodies in the same population that is affected by Zika virus. Similarly, it was not known if antibodies to Japanese encephalitis virus could cause antigenic interference in developing immunity to Zika virus and it was an interesting thought to study the same. The proposed work also provides an insight to any possible immune interference caused by prevalent JE and CHIKV antibodies to candidate Zika vaccine.

In current invention, candidate Zika virus vaccines have been developed and tested for potency with various formulations to elicit the appropriate level of immune response to protect against Zika virus disease. As there was no significant antigenic interference to Zika induced immune response by Japanese encephalitis virus vaccine and Chikungunya virus vaccine when co-administered or combined as a combination vaccine, the formulations were effective in eliciting high level of neutralizing antibodies capable of conferring protection against each of the viruses

OBJECTS OF THE INVENTION

One object of the invention is to provide stable immunogenic compositions for prophylaxis and treatment of Zika virus infections.

Another object of the invention is to provide methods for adaption and growth of Zika virus in Vero cells.

Another object of the invention is to provide methods for the preparation of inactivated Zika virus vaccine and purification of Zika virus bulk antigen.

One more object of the invention is to provide methods for Zika virus inactivation by chemical means with formalin, beta propiolactone and hydrogen peroxide

Yet another object of the invention is to provide methods for Zika virus inactivation by physical means such as heat, gamma irradiation and ultraviolet light

Yet another object of the invention is to provide methods for the preparation and formulation of recombinant Zika virus antigens comprising the prME protein and testing for immunogenicity in animals

A further object of the invention is to provide methods for formulations of Zika virus antigens with different adjuvants and estimation of immune response to the formulations in animals.

Yet another object of the invention is to provide kinetics of immune response to single dose, two and three doses of formalin and BPL inactivated Zika virus vaccine in animals.

One more object of the invention is to provide immunogenic compositions for prophylaxis of Zika and Chikungunya virus infections

Another object of the invention is to provide immunogenic compositions for prophylaxis of Zika, Chikungunya and Japanese encephalitis virus infections.

SUMMARY OF THE INVENTION

- The present invention is directed to compositions and methods of manufacture of vaccine formulations for prophylaxis and treatment of Zika virus infections as well as infections caused by other arboviruses such as Chikungunya virus and Japanese encephalitis virus.
- In one aspect, the invention is directed to vaccine compositions for prophylaxis and treatment of Zika virus infections, wherein the said compositions comprise Zika virus antigens in immunogenic compositions and may also comprise one or more arbovirus antigens such as Chikungunya virus and Japanese encephalitis virus antigens, along with suitable adjuvants and excipients.
- In another aspect, the invention is directed to a method of obtaining the vaccine formulations by a process which comprises:
    (a) Using Vero cell line as cell substrate for Zika virus culture
    (b) Scaling up the Zika virus culture upto a harvest volume of 10L
    (c) Inactivating the virus culture
    (d) Purifying the virus culture
    (e) In another aspect, recombinant cloning and expressing Zika virus pRME protein.

- In one embodiment of the invention, Vero cell line was used as the cell substrate for culture of Zika virus and was grown in culture medium with or without the use of serum.
- In another embodiment of the invention, the Zika virus was adapted by repeated serial passage in Vero cells to obtain higher titers.
- In another embodiment of the invention, Zika virus was passaged in C6/36 Ae. Albopictus cells followed by growth in Vero cells to increase the titer.
- In another embodiment of the invention, processes for scaling up the virus culture and further purifying the scaled up virus cultures is disclosed, wherein the harvest volume was about 8-10 L. The viral harvest was inactivated using various methods. The virus was then purified.

- In another embodiment of the invention, inactivation method is selected from a group of Formalin inactivation, Beta Propiolactone (BPL) inactivation, heat inactivation, UV inactivation, gamma inactivation, in the presence or absence of virus stabilizing agents and amino acids.
- In a preferred embodiment of the invention, amino acids were selected individually or in combination, from a group L-Histidine, L-Glutamic acid, L-Glycie and L-Aspartic acid and L-Glutamine and human serum albumin.
- In another preferred embodiment of this invention, the purification method is selected by use of cellufine sulphate, DEAE-Sephadex CM-sephadex with salt gradient, by gel filtration on Captocore-700, Sepharose CL-4B, ceramic hydroxyapatite column with gradient of 0.2M to 0.8M phosphate followed by diafiltration, and ultracentrifugation on a 20-60% sucrose gradient, most preferably by Capto core 700 column.
- Another embodiment of the invention is directed to recombinant cloning and expression of Zika virus prME protein is provided. The method of recombinant cloning utilizes a site specific transposition of the expression cassette with the cloned inserts into a baculovirus shuttle vector propagated in *E.coli* and expressed in insect cells.
- In another embodiment of the invention, vaccine formulations are provided. The vaccine may comprise of one or more Arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis viruses.
- In another embodiment, adjuvants can be selected from a group of aluminium salts, inulin, algammulin, combination of inulin and aluminium hydroxide, monophosphoryl lipid A (MPL), resiquimoid, muramyl dipeptide (MDP), N-glycolyl dipeptide (GMDP), poly IC, CpG oligonucleotide, resiquimod, aluminium hydroxide with MPL, any water in oil emulsion, any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitan trioleate, alpha-tocopherol, cholecalciferol or any of the analogues and derivatives of the molecules thereof, or calcium phosphate or any combination of the adjuvants.
- In another embodiment of the invention, the formulations is prepared with excipients and preservatives.
- In another embodiment of the invention, stabilizing agents in the vaccine formulation were used individually or in combinations of sorbitol, L-glycine, mannitol, L-glutamic acid and human serum albumin in various concentration was used to study the same.
- In another embodiment of the invention, the potency of the vaccine formulations have been tested in animal models to show complete protection from viremia over a wide range of dosage.
- In another embodiment of the invention, the combination vaccine formulations were also effective in providing adequate protection against Japanese Encephalitis as well as Chikungunya viruses.
- In another embodiment of the invention, the antisera confers passive immunity in rabbit against Zika virus infection to offer complete protection against viremia while viremia was detected in the control animals that persisted up to 6 days after virus challenge.
- In another embodiment of the invention, the candidate inactivated Zika virus vaccine can be administered either as a single dose, or in two or more doses by intramuscular route

- In another embodiment of the invention, assays for neutralizing antibody titers were conducted to check the antibody levels against vaccine formulations of the present invention which has shown to elicit high level of neutralizing antibodies.
- In another embodiment of the invention, cross neutralization studies exhibited that inactivated vaccine formulations of the present invention would be equally protective and potent against any Zika virus strain.
- In another preferred embodiment of the invention, antibody titers to both BPL inactivated and formalin inactivated Zika vaccine formulations were higher with aluminium hydroxide than with antigens alone.
- In another embodiment of the invention, quality of antibody responses to the vaccine formulations of the present invention by antibody avidity assays indicated that high affinity antibody were developed over time with booster doses.

Accordingly, the invention provides a stable vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, said antigens being formulated with or without an adjuvant in pharmaceutically acceptable buffer, wherein the vaccine composition elicits protective immune response to each of the viruses in mammals. The Zika virus antigen of the composition is effective for treatment, diagnosis and prophylaxis against any genotype/genotypic variants/strains of Zika virus, wherein the composition is effective against any genotype/genotypic variants/strains/synthetic Zika viruses that share anywhere between 50% to 100% identity at the amino acid level in any region of the genome. The composition of the invention comprises Zika virus antigens of any genotype/genotypic variant/strains/synthetic Zika virus, wherein the antibodies against any of the aforementioned Zika virus types cross neutralizes the homologous virus or any heterologous Zika virus strain that shares at least 50% -100% amino acid identity in any region of its whole genome, particularly the envelope E protein.

The antigens of Zika virus, Chikungunya virus and Japanese encephalitis virus of the composition are inactivated whole virion (virus) antigens. Whereas, the Zika and Chikungunya virus antigens are purified recombinant antigens.

The Zika virus antigen of the invention is prepared using Vero cells as cell substrate by adapting the virus to Vero cells.

The Zika virus antigen of the composition of the invention is a purified and concentrated antigen obtained from one or more methods selected from:

a. ultracentrifugation;
b. density gradient centrifugation;
c. clarification of the viral harvest using membrane filtration, followed by purification by column chromatography; and
d. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.

Wherein the purification by column chromatography comprises gel filtration, mixed mode resin column chromatography, ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography. The column chromatography elutes majority of the virus antigen in the flow through such as Capto Core 700, most preferably Capto Core 700 wherein the virus sample is purified on Capto Core 700 column and is eluted in the flow through.

The Zika virus of the composition is inactivated by at least one or more of a chemical inactivating agent, a physical inactivating agent and an irradiating agent, wherein the inactivation of Zika virus is carried out before or after purification of the virus. In an exemplary embodiment the Zika virus is inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.

In one preferred embodiment the Zika virus is inactivated by any one of the following methods selected from:

a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8^0C to 37^0C, preferably 25 ± 3^0C, for at least 1 to 7 days;
b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2^0C to 8^0C for at least 10 to 30 days;
c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs at temperatures ranging from 8^0C to 30^0C, preferably 25 ± 3^0C, for 48 hours;
d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2^0C to 8^0C for at least 3-7 days;
e. A combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL :virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15^0C to 30^0C, preferably 25 ± 3^0C ;
f. Hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30^0C for 5 minutes to 120 minutes.

In one embodiment, the inactivation of the Zika virus by irradiating agent comprises inactivation by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ^{60}Co source.
In another embodiment, the inactivation of the Zika virus by irradiating agent comprises inactivation by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

In a further embodiment, the virus is inactivated by heat treatment at a temperature between 50°C to 65°C for 30 min up to 2 hrs.

The buffer used in the invention may be selected from the list comprising of phosphate buffer, citrate buffer, phosphate citrate buffer, borate buffer, tris(hydroxymethyl)aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents, wherein phosphate buffer is sodium phosphate buffer at concentration of 5 mM up to 200 mM of phosphate ions of any pH between 6.50 to pH 9, and optionally containing sodium chloride at a concentration of 50 to 200 mM. The buffer maintains the pH in a liquid composition above pH 6.5, preferably above pH 7.0 throughout the bioprocess from viral culture up to preparation of purified inactivated virus bulk.

In one embodiment, the inactivation of Zika virus is carried out in the presence of a stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof. However, in one preferred embodiment, the stabilizing agent may be selected from:

a. 2% sorbitol and 1% L-glycine;

b. 1% sorbitol and 0.5 % L-glycine;
c. 1% mannitol and 0.5% L-glycine;
d. 1% mannitol and 0.5% L-glutamic acid; and
e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

In an exemplary embodiment, the inactivation of Zika virus comprises inactivation of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.

The purified recombinant Zika virus of the invention comprises antigens of Zika virus comprising the envelope (E) protein, membrane (M) protein and optionally the non-structural 1 (NS1) protein as vaccine antigens for eliciting immune response for prophylaxis of Zika virus infections, wherein the Zika virus has the structural protein sequences as disclosed in SEQ. ID No. 3 and SEQ. ID No.4 corresponding to nucleotide sequences of SEQ ID. No. 1 and SEQ ID No.2 respectively, for use as vaccine antigens against Zika virus infections caused by genotypes or variants thereof. The Recombinant DNA constructs comprises a (i) vector (ii) at least one nucleic acid fragment corresponding to SEQ ID NO.1 or SEQ ID NO. 2 encoding the amino acid sequence of the proteins of SEQ ID NO.3, SEQ ID NO.4 respectively which is applicable to any Zika virus protein sequences that share at least 70% amino acid identity to the aforementioned SEQ ID NO. 3 and SEQ ID NO.4. The composition of the invention comprises recombinant DNA construct, wherein the vector is an eukaryotic plasmid vector being cloned in a eukaryotic host such as baculovirus for expression in insect cells as virus like particles (VLPs).

The recombinant protein of Zika virus is obtained by the process comprising the steps of:
a. transfecting the recombinant plasmid DNA in insect cells;
b. harvesting the cells and isolating the recombinant protein therefrom;
c. purifying the protein by a method selected from ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation and fractionation with salt.

The structural antigens of Zika virus are expressed in any prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

The vaccine composition of the invention is obtained by a process wherein neutralizing antibodies are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

The vaccine composition of the invention may further comprise an adjuvant, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil

emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. In one preferred embodiment the composition comprises aluminum hydroxide in a concentration range of 0.1 mg to 1.5 mg of aluminum per vaccine dose, preferably 0.25 mg to 0.5 mg aluminum per vaccine dose.

The adjuvant of the composition of the invention confers mucosal immunity and systemic immunity when administered in mammals.

The vaccine composition with Zika virus antigen is administered at any dose ranging from 0.125 µg to 100 µg per dose with or without an adjuvant, either as a single dose or in two or more doses to elicit an immune response in a mammal.

In one embodiment the invention provides a method of eliciting a protective immune response in mammals including humans comprising administering the vaccine composition of claim 1 by any route comprising intramuscular, intradermal, subcutaneous, intravenous, oral, intranasal or transcutaneous routes.

The composition of the invention may be administered by any method comprising needles and syringes including pre-filled syringes, microneedle patch, needle-free patch, inhalation and nasal sprays.

The invention also provides a method of *in vitro* or *in vivo* use of the Zika virus antibodies of the composition for preparation of immunodiagnostic and immunotherapeutic agents for Zika virus infections.

In one embodiment the vaccine composition comprises Zika virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses, wherein the Zika virus antigen and Japanese encephalitis virus inactivated antigens are present in the combination vaccine at concentrations ranging 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

The adjuvant may be selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. In one preferred embodiment, the adjuvant is aluminium hydroxide with 0.25 mg to 1.0 mg of aluminium content per vaccine dose.

In another embodiment, the vaccine composition comprises Zika virus and Chikungunya virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses, wherein Zika and Chikungunya virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

The adjuvant may be selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. In one preferred embodiment, the adjuvant is aluminium hydroxide at 0.25 mg to 1.5 mg of aluminium content per vaccine dose.

In another embodiment, the vaccine composition comprises Zika virus, Chikungunya virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses, wherein Zika virus, Chikungunya virus and Japanese encephalitis virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

The adjuvant may be selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. Preferably, the adjuvant is aluminium hydroxide at 0.25 mg to 1.0 mg of aluminium content per vaccine dose.

The vaccine composition of the inveniton optionally comprises 2-phenoxyethanol preservative at a concentration of 2.5 to 5 mg/mL.

The vaccine composition when administered in a single dose or in two or more doses in mammals elicits both Th1 and Th2 immune response against any of the arbovirus antigens comprising Zika Virus, Chikungunya virus and Japanese Encephalitis virus and is suitable for administration to humans.

In one embodiment the invention provides a method for preparation of a vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, the method comprising one or more steps of inactivation,

producing recombinant protein, expressing structural antigens, purification and concentration of the virus antigen wherein said purification and concentration of Zika virus comprises one or more steps selected from:

a. ultracentrifugation ;
b. density gradient centrifugation;
c. clarification of the viral harvest using membrane filtration;
d. purification by column chromatography;
e. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.

The column chromatography method comprises gel filtration, mixed mode resin column chromatography, any ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography, wherein the column chromatographic method elutes majority of the virus antigen in the flow through such as Capto Core 700, most preferably Capto Core 700wherein the virus sample is purified on Capto Core 700 column and is eluted in the flow through.

The Zika virus is inactivated by one or more inactivating agents selected from a chemical inactivating agent, a physical inactivating agent and an irradiating agent.

The preparation method comprises inactivation of Zika virus which may be carried out before or after purification of the virus, wherein the Zika virus may be inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.

In one embodiment, the preparation method comprises inactivation of the Zika virus bulk which is inactivated by any one of the following methods selected from:

a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8^0C to 37^0C, preferably 25 ± 3^0C, for at least 1 to 7 days;
b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2^0C to 8^0C for at least 10 to 30 days;
c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs, if not more, at temperatures ranging from 8^0C to 30^0C, preferably 25 ± 3^0C, for 48 hours;
d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2^0C to 8^0C for at least 3-7 days;
e. a combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL :virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15^0C to 30^0C, preferably 25 ± 3^0C ;
f. hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30^0C for 5 minutes to 120 minutes.

In embodiment of preparation method, the virus is inactivated by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ^{60}Co source.

In another embodiment of preparation method, the Zika virus is inactivated by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

In another embodiment of the preparatory method, the Zika virus is inactivated by heat treatment from 50°C to 65°C for 30 min up to 2 hrs, preferably, 65°C for 1 hr.

In one embodiment of the preparation method, the inactivation is carried out in the presence of stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof. In one preferred embodiment the stabilizing agent is selected from:

a. 2% sorbitol and 1% L-glycine;
b. 1% sorbitol and 0.5 % L-glycine;
c. 1% mannitol and 0.5% L-glycine;
d. 1% mannitol and 0.5% L-glutamic acid; and
e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

The inactivation methods described hereinabove are applicable to Zika virus of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.

In one embodiment the invention discloses a method of producing the recombinant protein comprising the steps of:

a. transfecting recombinant plasmid DNA in insect cells;
b. harvesting the cells and isolating the recombinant protein therefrom;
c. purifying the protein by at least one of the methods comprising of ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation, fractionation with salt.

In another embodiment, the invention discloses method of expressing the structural antigens of Zika virus comprising expression system is any prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

In another embodiment the invention discloses a method wherein the method comprises neutralizing antibodies that are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

The vaccine composition of the invention may be administered in a prime boost strategy, wherein the prime is the candidate inactivated vaccine and the boost is either the same vaccine or any other vaccine such as DNA vaccine, Chimeric Zika virus vaccine, virus like particles, deactivated Zika vaccine, live attenuated virus vaccine, recombinant subunit vaccine, vectored vaccine or any vaccine derived from synthetic Zika virus, wherein the neutralizing antibodies in each of them are elicited against Zika virus Envelope protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Fig. Purified Zika virus bulk in 12.5% SDS-PAGE gel detected by silver staining. The Envelope (E) protein and the Membrane (M) proteins are the major proteins detected in the purified antigen

Figure 2: Fig.2A – Inactivation kinetics of Zika virus by formalin at concentrations ranging from 1:1000 v/v of formalin: virus up to 1:4000 v/v of formalin: virus at 25±3^{0}C. Fig.2B – inactivation kinetics of Zika virus by beta-propiolactone at concentrations ranging from 1:1000 up to 1:3500 v/v of BPL: virus at 25±3^{0}C. In both the inactivation procedures, 1% sorbitol ad 0.5% L-glycine (final concentration) were added as stabilizers, which had no effect on the inactivation kinetics. The inactivated samples were serially amplified three times *in vitro* in Vero cells, and assayed at the end of three passages by TCID50.

Figure 3: Fig.3A: The ~2.1 kb Zika virus prME gene of SEQ ID NO.1 was amplified by gene specific primers for initiating cloning in pFastBac vector for expression in insect cells. Fig.3B: The Sf9 cell lysate was probed by Western for detection of expression of the prME protein using Zika rabbit polyclonal antisera by standard procedures. The envelope protein of ~ 55 kD could be detected as the major band.

Figure 4:Estimation of neutralizing antibody titers elicited by Zika vaccine formulations with different adjuvants. Adjuvants are abbreviated as follows: pIC (polyIC); C- cholecalciferol; MPL (lipid A; monophosphoryl); RP (resiquimoid + polyIC); RM (resiquimoid + OWEM2); I (inulin); OWEM2 (oil in water emulsion 2); AI (aluminum hydroxide + inulin); MDP (muramyldi peptide); OWEM1 (oil in water emulsion 1). No significant antibody titers could be detected in the respective control groups and hence not depicted in the figure. In all cases, 10 µg of two doses of Zika vaccine antigen was formulated for administration in Balb/c mice by IM route.

Figure 5: Fig. 5A:Estimation of neutralizing antibody titers by $PRNT_{50}$ in dose ranging studies from 0.125 µg up to 40 µg per dose of the aluminum hydroxide adjuvanted formalin inactivated Zika virus vaccine administered by IM route in Balb/c mice in two doses. Fig.5B: the vaccinated animals were challenged intravenously with 10e5 PFU /animal of the Zika virus strain 7 days after the booster dose, and viremia was monitored every 24 hours for 7 days (depicted in the graph for 6 days). All the animals showed complete protection from viremia whereas the animals administered the placebo control showed viremia that persisted up to 6 days. The infectious virus was estimated in the blood samples by $TCID_{50}$.

Figure 6: Virus challenge in 4-6 week old Balb/c mice after administration of 1 µg to 40 µg of BPL inactivated, alum adsorbed Zika virus vaccine. Animals of all the vaccine dose groups and the placebo group were challenged with 10e5 PFU of Zika virus MR766 strain 7 days after administration of the booster dose. Viremia was monitored every 24 hours after virus challenge, and the titers of infectious particles in blood were estimated by $TCID_{50}$. The candidate Zika vaccine offered complete protection from virus challenge at all dose groups.

Figure 7: Passive immunization offered complete protection against viremia and infectious virus could not be detected by $TCID_{50}$ in the animals that received Zika rabbit polyclonal antisera intraperitoneally and challenged 24 later with 10e5 PFU of Zika virus. Infectious virus particles could not be detected by $TCID_{50}$ in the blood, when monitored every 24 hours for 6 days, whereas the control animals that received equal volume of PBS showed persistent viremia up to 6 days when challenged with the same dose of the virus.

Figure 8: Formalin inactivated, alum adsorbed Zika virus vaccine antisera from vaccinated mice neutralized the homologous MR766Zika virus strain (Fig.8B) and cross neutralized the heterologous Asian genotype FSS 13025strain (Fig.8A) with equal efficiency with $PRNT_{50}$ titers of 18105 and 18325 respectively. The values for the placebo (alum only) are also depicted in the graph alongside.

Figure 9:Antibody titers expressed as log10 of reciprocal of serum dilutions from the dose ranging studies with (Fig.9A) single dose (Fig.9B) two doses (Fig.9C) three doses of formalin inactivated vaccine administered in 4-6 week old Balb/c mice as described in Example 7. Fig.9D is antibody titers with a single, two and three doses of 10 µg of vaccine antigen without alum. All values are expressed as Geometric Mean Titers with 95% CI. In 9A-9D, individual animal data is plotted. Zika virus antigen was immunogenic even without an adjuvant. Data from other dose ranges were estimated but not included in the graph.

Figure 10: High affinity antibodies could be elicited by single dose of formalin inactivated alum adsorbed Zika virus vaccine in Balb/c mice even at low doses of the vaccine antigen up to 1 µg. Antibody avidity was expressed as avidity index and estimated by methods described in Example 12.

Figure 11: Estimation of Th1 cytokines(Fig.11A) IFN gamma, and (Fig.11B) IL-2 in mice vaccinated with Zika vaccine formulations with different adjuvants. In all cases it was 10 µg of vaccine antigen per dose. Adjuvants are abbreviated as follows: pIC (polyIC); C-cholecalciferol; MPL (lipid A; monophosphoryl); RP (resiquimoid + polyIC); RM (resiquimoid + OWEM2); I (inulin); OWEM2 (oil in water emulsion 2); AI (aluminum hydroxide + inulin); MDP (muramyl di peptide); OWEM1 (oil in water emulsion 1) as described in Example 5. Oil based adjuvants and polyIC elicited a strong Th1 response compared to other adjuvants tested.

Figure 12:Estimation of Th2 cytokines (Fig.12A)IL-4, and (Fig.12B) IL-10, in mice vaccinated with Zika vaccine formulations with different adjuvants. In all cases it was 10 µg of vaccine antigen per dose. Adjuvants are abbreviated as follows: pIC (polyIC); C-cholecalciferol; MPL (lipid A; monophosphoryl); RP (resiquimoid + polyIC); RM (resiquimoid + OWEM2); I (inulin); OWEM2 (oil in water emulsion 2); AI (aluminum hydroxide + inulin); MDP (muramyl di peptide); OWEM1 (oil in water emulsion 1) as described in Example 5. Oil based adjuvants and polyIC elicited strong Th2 response in addition to Th1 response.

DETAILED DESCRIPTION OF THE INVENTION

This disclosure concerns formulation of immunogenic compositions. The invention discloses in particular, preparation and formulation of vaccine antigens of Zika virus in monovalent compositions and in combination with other arboviruses such as Chikungunya and /or Japanese encephalitis viruses. In particular, the invention discloses compositions for prophylaxis and treatment of Zika virus infections.

One aspect of the invention is that the methods of preparation, formulation and use of Zika antigens as vaccine for eliciting immune response is applicable to any genotype, genotypic variants or any strain of Zika virus wherein one genotype of Zika virus cross neutralizes a heterologous strain efficiently. The Zika virus can be selected from Asian, West African or East African genotype of the virus. Therefore, the methods described in the current invention herein are applicable to Zika virus of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein and the M protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome. A chimeric virus has the nucleic acid of a heterologous virus and nucleic acid of Zika virus.

In the context of the immunogenic compositions disclosed herein, in particular the bulk antigen used for preparation of immunogenic compositions, the methods of preparation, formulations and use of Zika vaccine antigens are applicable to any of the aforementioned Zika virus types, that share at least 50% amino acid identity and up to 100% amino acid identity across any region of the genome. In the context of the immunogenic compositions disclosed herein, sequence of Zika virus MR766 strain of African genotype (SEQ ID NO.5 for genomic nucleotide sequence and SEQ ID NO.6 for complete ORF) shares more than 96.5% amino acid identity in the structural Envelope protein with the Asian genotype strain FSS13025 and whose sequence is disclosed in SEQ ID NO.7 and SEQ ID NO.8 for the nucleotide and protein sequences respectively. Vaccine antisera of the MR766 strain cross neutralized the FSS13025 strain with 100% equivalent potency as the homotypic MR766 strain. Also in the context of the disclosure herein, Zika virus prME (SEQ ID NO.3) antisera efficiently cross neutralized the MR766 strain confirming that all Zika viruses are serotypically similar. In the context of the disclosure herein, the vaccine methods developed using any one of the Zika virus strains is applicable to homologous and any heterologous Zika virus strains for use as candidate vaccine.

A cell line that can be propagated *in vitro* in culture can be used as a host for Zika virus culture. For propagating Zika virus strains, preferably permissive cells which allow the virus to grow well are selected. For example, diploid cell lines such as MRC-5 and WI-38, and serially passaged cell lines such as Vero, BHK-21, CHO cells etc. can be used. For example, Vero cells (ATCC No. CCL-81), BHK-21 (ATCC No. CCL-10), C6/C3 (ATCC No. CRL-1660) etc. can be used. In a preferred embodiment, one such cell line used in the current invention is Vero cells (ATCC No. CCL-81) which has been validated for use as a host cell for vaccine production. The validated Vero cell lines conforms to the Requirements for Biological Substances No.50 regarding requirements for use of cells for the production of biologicals recommended by the World Health Organization (WHO) thereby confirming these cell lines as qualified for producing a vaccine (WHO Technical report Series, No. 878, pp 19-52, 1998).

In one aspect of the invention, the method of adaptation of Zika virus to Vero cells increases the virus titer. Zika virus passaged repeatedly in Vero cells increases the virus titer. In the context of virus growth in Vero cells disclosed herein, Zika virus passaged initially in mouse brain or *Ae.albopictus* C6/36 cells (ATCC No. CRL-160) and then adapted to Vero cells increases virus titers suitable for vaccine production.

For maintenance in cell culture of the above-mentioned cell lines, Vero cells in particular, stationary culture in monolayers, perfusion system culture, shake flasks, roller tube/bottle culture, suspension culture with and without microcarriers, cell factories and cell stacks, bioreactors and disposable bioreactors, wave bioreactors and the like can be adopted. For example various types of microcarriers are commercially available. Commercially available animal cell culture devices can be used to facilitate the growth of cells to high cell density.

In one aspect of the invention and disclosed herein, the Zika virus is purified for use as candidate vaccine. Purification is achieved by a combination of both physical and chemical methods either before or after inactivation of the virus. Physical methods include any of the following techniques but not limited to: ultracentrifugation, density gradient centrifugation, ultrafiltration, diafiltration and concentration using semi-permeable membranes with suitable molecular cut-off sizes. Purification through chemical means employs methods such as adsorption/desorption through chemical or physiochemical reactions such as ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, gel filtration chromatography such as for example Captocore700^{TM}, hydroxyapatite matrix, salting with inorganic salts, one such example being ammonium sulphate.

In a preferred embodiment, the virus is purified on Capto core 700 (GE Healthcare Life Sciences) column chromatography. Inactivation of the virus is achieved either before purification or after purification on Capto core 700 column. The virus harvest before Capto core 700 column can be clarified using membrane filters with different pore sizes, preferably not less than 0.45 µM low protein binding membrane. In a preferred embodiment, the virus harvest can be clarified with a dual membrane of two different pore sizes, for example 1.2 µM followed by 0.45 µM, or 0.8 µM followed by 0.45 µM. The clarified virus harvest is suitable for purification on Capto Core 700 column. The buffers used for purification on Capto core 700 is of optimal pH and ionic strength to maximize the binding of the impurities on the column and elute the virus in the flow through. The virus sample is further concentrated by diafiltration before or after virus inactivation. Diafiltration of the virus sample after inactivation removes the virus inactivating agent from the bulk antigen,and is suitable for formulation.

In one embodiment of the invention, Zika virus in inactivated (killed) for use as a vaccine antigen. Inactivation can be carried out either before or after purification of the virus. In a preferred embodiment, inactivation of Zika virus is carried out after purification of the virus.

Zika virus can be inactivated either by heat, gamma irradiation, ultraviolet light or by chemical means. In a preferred embodiment disclosed herein, Zika virus is chemically inactivated. Chemical inactivating agents were selected from the following list which includes but is not limited to: formalin, beta-propiolactone, glutaraldehyde, N-acetylethyleneimine, binary ethyleneimine, tertiary ethyleneimine, ascorbic acid, caprylic acid, psolarens, detergents including non-ionic detergents etc. wherein the chemical inactivating agent is added to a virus suspension to inactivate the virus.

In a preferred embodiment of the invention, the chemical inactivating agent selected is formalin and/or beta propiolactone (BPL). Formalin is used at any concentration ranging from 1:1000 to 1:4000 v/v of formalin: virus. Beta propiolactone is used at any concentration ranging from 1:1000 to 1:4000 v/v of BPL: virus. The temperature and duration of inactivation is optimized to complete virus inactivation with minimal adverse effect on immunogenicity. This can be achieved with shorter duration of exposure with minimum quantity of the inactivating agent. In the context of virus inactivation, the disclosure herein describes the concentration, temperature and time of exposure of Zika virus to formalin and BPL. In the preferred embodiment of the invention, the inactivation temperature is 25 ± 3^0C, most preferably 22^0C for 7 days. At lower temperatures of 2^0C to 8^0C, the duration of formalin exposure is longer than 7 days to achieve complete virus inactivation at the aforementioned concentration ranges. Duration of virus exposure to formalin can be reduced to below 48 hours by increasing the temperature of exposure up to 37^0C. Hence, effective formalin inactivation of Zika virus can be achieved at any concentration range of formalin from 1:1000 v/v formalin: virus up to 1:4000 v/v formalin: virus by choosing any temperature range from 2^0C to 37^0C and varying the exposure time from 24 hours to more than 10 days at any of the aforementioned concentrations, time and temperature of exposure.

In one embodiment of the disclosure, BPL is used as virus inactivating agent for Zika virus. In a preferred embodiment of the invention, BPL is used at concentrations ranging from 1:1000 v/v BPL: virus up to 1: 4000 v/v BPL: virus. At lower temperatures of 2 to 8^0C, the duration of BPL exposure is preferred for 3 to 7 days to achieve complete virus inactivation at the aforementioned concentration ranges. Duration of virus exposure to BPL can be reduced to 48 hours or below by increasing the temperature of exposure up to 25 ± 3^0C or even up to 37^0C. Hence, effective BPL inactivation of Zika virus can be achieved by choosing any concentration range of BPL from 1:1000 v/v BPL: virus to 1:4000 v/v BPL: virus by choosing any temperature range from 2 to 37^0C and varying the exposure time from 24 hours to more than 10 days at any of the aforementioned concentrations, time and temperature of exposure.

One of embodiments of the current invention disclosed herein is the use of a combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 v/v of BPL: virus for 24 hours followed by formalin inactivation at 1: 3000 v/v formalin: virus for 24to 48 hours at 15^0C to 30^0C, preferably 25 ± 3^0C. The use of BPL and formalin combination for Zika virus inactivation is that the mechanism of inactivation being different for formalin and BPL, their combined used reduces their overall concentration and exposure to both the inactivating agents, and also the use of low concentrations of formalin promotes stability of the virus bulk by promoting cross linking of virus epitopes. In another embodiment of the invention, hydrogen peroxide is used for inactivating the Zika virus at concentrations ranging from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20^0C to 30^0C for 5 to 120 minutes, if not more.

An embodiment of the current invention discloses the use of the prME antigen of Zika virus as the candidate vaccine antigen to elicit immune response against the Zika virus. The disclosure is applicable to any method of vaccine design wherein the prME or the E protein is expressed in such a manner that the neutralizing Zika antibodies are directed against the said antigens. In a preferred embodiment of the invention, the prME protein is expressed as recombinant virus like particles (VLP) in baculovirus mediated expression in insect cells. Anyone skilled in the art will derive additional embodiments using the above disclosure to design a vaccine candidate using the prME protein as the target Zika antigen such as a DNA

vaccine, Virus like particles comprising prME proteins, subunit vaccine comprising the Envelope (E) antigen, live vectored vaccines, chimeric vaccines using the Zika prME on a heterologous nucleic acid backbone wherein in all the above the anti-Zika antibodies are directly against the E protein.

In the current invention disclosed herein, are immunogenic compositions comprising purified recombinant Zika virus antigens comprising the envelope (E) protein, membrane (M) protein and optionally the non-structural 1 (NS1) protein as vaccine antigens for eliciting immune response for prophylaxis of Zika virus infections. In a preferred embodiment, the use of the Zika virus having of the prME gene of sequences SEQ ID NO.1 and SEQ ID NO.2 encoding the structural protein of SEQ. ID NO. 3 and SEQ. ID NO.4 respectively, wherein the expressed and purified prME protein can be used as vaccine antigen for prophylaxis of Zika virus infections.

In a preferred embodiment, Zika virus prME gene is used to generate a recombinant gene construct that can be used to express the prME protein in prokaryotic or eukaryotic expression systems as virus like particles (VLPs), preferably baculovirus mediated expression in insect cells. The methods disclosed herein are applicable to any Zika virus strain that share at least 70% amino acid identity to the aforementioned SEQ ID NO. 3 and SEQ ID NO.4

An embodiment of the current disclosure is the choice of pharmaceutically acceptable buffer throughout the bioprocess wherein the buffering agent is selected from a list consisting of any one or more of the following, but not limited to: phosphate buffer; citrate buffer; phosphate citrate buffer; borate buffer; tris(hydroxymethyl)aminomethane (Tris) containing buffer; succinate buffer; buffers containing glycine or histidine as one of the buffering agents. In the most preferred embodiment, phosphate buffer is used, wherein phosphate buffer is sodium phosphate buffer at concentration of 5 mM up to 200 mM of phosphate ions, preferably 10 mM to 100 mM phosphate buffer, most preferably 10 mM to 50 mM phosphate buffer of any pH above 6.50 to pH 9, preferably pH 6.8 to pH 7.8 is used for the upstream and downstream processes. In a preferred embodiment, 10 mM sodium phosphate buffer of pH 7.4±0.2 is used in the preparation of the purified inactivated vaccine bulk of Zika virus antigen, and optionally containing sodium chloride at a concentration from 50 to 200 mM. In another preferred embodiment, sorbitol and L-glycine are optionally added to a final concentration of 1% and 0.5% respectively.

An embodiment of the current invention also discloses the choice of adjuvants that is compatible for formulation with Zika virus antigen.

The antigenic compositions of Zika virus as monovalent vaccine, and with Chikungunya virus and Japanese encephalitis viruses in combination vaccine were formulated in pharmaceutically acceptable carrier for immunization. The use of adjuvant(s) can reduce the amount of antigen required in the formulation Furthermore, for adjuvanted vaccine formulations, suitable adjuvant(s) were selected from the following list, which includes but is not limited to: alum such as aluminum hydroxide, aluminum phosphate, or amorphous aluminum sulphate phosphate; calcium phosphate; inulin of any polymorphic form, preferably gamma inulin; adjuvants containing inulin in combination with other organic and inorganic compounds such as aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate and calcium phosphate; liposomes, chitosan and complex carbohydrates such as dextran, dextrins, starch, mannans and glucomannans, galactomannans, beta-glucans, heparin, cellulose, hemicellulose, pectins and pectinates, lectins and any other carbohydrates

either synthetic or derived from any source, any biodegradable and biocompatible polymers, such as poly lactide and polylactide co-glycolides, (PLG or PLGA); any emulsions including but not limited to oil in water emulsions one such example being squalene or squalene analogues containing oil in water adjuvants, oil in water emulsions containing vegetable oils; any water in oil emulsion; liposomes prepared with cholecalciferol as one of the ingredients along with other lipid soluble compounds; liposomes of other compositions; RIBI adjuvant systems, saponins including but not limited to QS-21, QuilA, tomatine, ISCOMs, ISCOMATRIX etc, lipopeptides, glycopeptides and their analogues, resiquimoid, lipopolysaccharides, lipid A, muramyl dipeptides or their analogues and any peptide based adjuvants, oligonucleotides, any TLR ligands and their analogues as adjuvants, any cytokine, vitamins and non-toxic bacterial toxins, indeed any analogues of all the aforementioned adjuvants and combination of two or more of the aforementioned adjuvants or their analogues that are compatible in vaccine formulation(s). and tested for enhanced immunogenicity. In addition to the above, any other organic and inorganic substances that have good immunopotentiating activity are suitable to be used as adjuvant either singly or in adjuvant combinations to enhance the immunogenicity of the arboviral antigens. The use of adjuvant in the vaccine formulations can reduce the amount of antigen required.

In a preferred embodiment of the invention, aluminum hydroxide was used for dose ranging studies of both formalin and BPL inactivated Zika antigens as well in vaccine combinations of Zika, CHIKV and JEV vaccines due its safety profile for use in target population. Oil based emulsions and polyIC gave good immunopotentiating effect to Zika antigen when used as adjuvants. In one embodiment of invention, polyIC and other adjuvants that offer both systemic mucosal immunity is particularly advantageous for protection against disease caused by Zika virus infections. PolyIC and the oil based emulsions and the adjuvant combinations disclosed in the invention elicited both Th1 and Th2 responses estimated by the measurement of the Th1 and Th2 cytokines after vaccination.

In one embodiment of the current invention, a vaccine preservative is used in the vaccine formulations. The preferred embodiment is 2-phenoxy ethanol at a concentration of 2.5 to 5 mg per dose.

In one aspect of the current invention disclosed herein are the use of stabilizing agents selected from one or more of the following, but not limited to: lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid, human serum albumin and combinations thereof, at any suitable concentration that are used to confer stability during the inactivation of Zika virus by any of the aforementioned methods. In a preferred embodiment, the stabilizing agents are selected from any of the following combinations but not limited to: 2% sorbitol and 1% L-glycine; 1% sorbitol and 0.5 % L-glycine; 1% mannitol and 0.5% L-glycine; 1% mannitol and 0.5% L-glutamic acid; 1% sorbitol, 0.5% L-glycine, 1% human serum albumin. In a preferred embodiment, the combination of 1% sorbitol and 0.5% L-glycine and 1% mannitol and 0.5% L-glycine are preferred combinations, most preferably, 1% sorbitol and 0.5% L-glycine. One skilled in the art will recognize further embodiments based on the above disclosures.

Lyophilized formulations are one of the methods for preparation of vaccine product. Lyophilized preparations of Zika virus vaccine typically contain purified inactivated Zika virus, a sugar polyol, preferably sorbitol and mannitol, most preferably sorbitol in

combination with a glass forming sugar, which is preferably a disaccharide or an oligosaccharide. The preferred disaccharide is selected from the following list but is not limited to: sucrose, trehalose, maltose, mannose, lactose, raffinose, isomaltose, stachyose etc. the preferred embodiment of the disclosure is a combination of 1% sorbitol with 5% sucrose, 1% mannitol with 5% sucrose, and 3% sucrose and 2% trehalose, 1% mannitol with 1% L-glycine and or 2% trehalose. Any one of the ordinary skill in the art will devise further embodiments and based on the disclosures above.

The lyophilized formulations can be re-suspended in water for injection or an aqueous buffer that is pharmaceutically acceptable for administration. e.g. as an injectable liquid to a human subject. The lyophilized formulation can also be used as an inhalable powder which will be suitable for inducing mucosal immunity. Additionally the lyophilized formulation of Zika virus can comprise an adjuvant that confers mucosal immunity preferably from a list of those adjuvants tested in the current invention for Zika virus such as polyIC for example.

In the current invention, the disclosure provided herein on the optimal use of Zika virus antigen to elicit robust immune response, the vaccine antigen can be used at 0.10 μg up to 100 μg per dose, wherein the preferred embodiment is any concentration from 0.125 μg up to 40 μg per dose such that the administered vaccine doses elicit antibody titers measurable by assays such as ELISA and $PRNT_{50}$. The vaccine can be administered with and without an adjuvant as both the inactivated vaccine and the adjuvanted formulations elicit good immune response.

In yet another disclosure of the invention, the inactivated Zika vaccine candidate inactivated by any of the disclosed methods can be administered as a single dose or in two or more doses to elicit immune response. The methods disclosed in the invention provide the kinetics of immune response after each dose of the vaccine, at dose ranges from 0.125 μg up to 40 μg per dose that offers the flexibility of the choice of the vaccine dose range concentrations and number of doses to suit the target population for vaccination.

The route of vaccine administration can be by any route selected from, but not limited to intramuscular, intradermal, subcutaneous, intravenous, oral, intranasal and transcutaneous routes. In a preferred embodiment of the invention, the preferred route of vaccine administration is intramuscular (IM) route.

The vaccine formulations can be presented in glass vials and injected by needle and syringes, presented in pre-filled syringes in a ready to use presentation or administered by electroporation, microneedle patches, needle free patch, by inhalation or by nasal sprays.

The current invention discloses methods for preparation and use of formulations comprising one or more arbovirus antigens selected from a list that includes Zika virus, Chikungunya virus (CHIKV), and Japanese encephalitis virus (JEV). When used in vaccine combination, the vaccine can elicit immune response against each of the viruses present in a combination vaccine. In a preferred embodiment of the invention comprising a vaccine composition wherein Zika virus antigens and Japanese encephalitis virus antigens are present in a combination vaccine at concentrations ranging from 5 μg to 50 μg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or preferably with an adjuvant selected from the list of adjuvants disclosed in the current invention, preferably aluminum hydroxide with 0.25 mg to 1.5 mg of aluminum content per vaccine dose is disclosed. In yet another preferred embodiment of the invention a vaccine composition comprising

Chikungunya and Zika virus antigens in a formulation comprising 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or preferably with an adjuvant selected from the list of adjuvants disclosed in the current invention, preferably aluminum hydroxide with 0.25 mg to 1.5 mg of aluminum content per vaccine dose is disclosed.

In yet another preferred embodiment of the invention, a vaccine composition comprising Chikungunya, Zika and JEV virus antigens in a formulation comprising 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or preferably with an adjuvant selected from the list of adjuvants disclosed in the current invention, preferably aluminum hydroxide with 0.25 mg to 1.5 mg of aluminum content per vaccine dose is disclosed. The use of vaccine combination confers a distinct economical advantage for manufacture and distribution of vaccines, provided that immune response is elicited against each of the antigen in the formulation and no antigenic interference is observed to either of the antigen by the presence of an additional antigen. The vaccine antigens can either be administered from a single formulation or administered separately at the same time or in suitable time intervals so as to elicit an immune response to the cognate antigen.

The current invention also discloses the use of Zika virus antibodies for detection of Zika virus by ELISA or in any immunodiagnostic methods where the antibodies find an application for detection or diagnosis of Zika virus infections.

The current invention also discloses herein the use of Zika virus antibodies for prevention and treatment of Zika virus disease.

Abbreviations used in the invention: IM – intramuscular; mcg-microgram; TCID50- 50% Tissue Culture Infectious Dose; PFU – Plaque forming unit

EXAMPLES

Example 1: Zika Virus culture in Vero cells

Vero cell line (ATCC No. CCL-81) was used as the cell substrate for culture of Zika virus. Extensively characterized Vero cells obtained from BioReliance, USA was used in pilot scale production. Vero cells were grown in DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich Catalog # D5523 and used as per the manufacturer's instructions) or EMEM (Eagles Minimal Essential Medium) containing 5% fetal bovine serum (FBS) or New Born Calf Serum (NBCS) and incubated at 35^0C to 37^0C until reaching 80 – 100 % confluence of the monolayer. Post-infection, the same medium containing 1% serum was used, or alternatively the virus was cultured in Vero cells adapted to serum free medium. Zika virus also could be grown in MRC-5 cell monolayer which were prepared in growth medium consisting of EMEM buffered to neutral pH with Hepes buffer with 5% serum and statically incubated at 35^0C to 37^0C for 6 to 8 days. Zika virus was cultured routinely in Vero cells. Zika virus MR766 strain (ATCC VR-84) was adapted to Vero cells by direct inoculation in Vero cells. Alternatively, the virus was adapted in C6/36 *Ae. albopictus* cells twice by serial passages, and the Zika virus in culture supernatant from these cells was used to infect Vero cells. Serial passage of Zika virus in C6/36 cells cultured at 25^0C to 28^0C increased the virus titer higher than 10e8.0 $TCID_{50}$/mL or 10e8.0 PFU/mL. This also obviated the need for subsequent repeat passages in Vero cells to obtain high titers. Virus adaptation by this method is useful to achieve high titers and subsequent higher yield in production. After culture in C6/36 cells, the virus was serially plaque purified twice from Vero cells, and the virus from a single well

isolated plaque was amplified and extensively characterized to be free of adventitious agents (all known RNA and DNA viruses, bacteria, fungi, mycoplasma etc) using the NGS (Next Generation Sequencing) platform. The virus genomic RNA was sequenced by NGS platform, and complete nucleotide sequence of MR766 strain is provided in SEQ ID NO.5 and the corresponding deduced amino acid sequence is provided in SEQ ID NO.6. Sequencing showed the intact glycosylation site in the Envelope protein, which otherwise is lost if the cells are extensively passaged in mammalian cells. Zika virus produces cytopathic effect (CPE) in Vero cells, and at the optimal Multiplicity of Infection (MoI) and harvest conditions, virus titers above 10e8.5 TCID50/mL or 10e8.5 PFU/mL could be attained.

Example 2: Zika Virus Purification

For Zika virus culture at pilot scale, the virus culture was systematically scaled up from T-175 flasks to CS1 (cell stack 1), CS10 (cell stack 10) and CS40 (cell stack 40). Multiples of CS40 simultaneously infected with the virus at standardized MoI was used to scale up production. Use of multiples of CS40 enables quick and linear scale up to the desired volumes of production. The harvest volume from each CS40 was approximately 8-10 L. The virus was harvested at days 4-6 or whenever more than 90% CPE was achieved. Alternatively, disposable bioreactors under well standardized conditions of temperature 35^{0}C to 37^{0}C, pH not less than 7.0, and optimally at pH 7.4, dissolved oxygen at 45 to 75 ppm, preferably 60 rpm and an agitation of 240 to 280 rpm and optimally controlled in-flow and out-flow rate optimized according to the scale of the culture volume from 1L to 100L was used to increase the cell density and virus harvest. The viral harvest was clarified either by microfiltration or using dual filters with cut off of 1.2 µM and 0.45 µM. The clarified viral harvest was then passed through Capto Core700 column (GE healthcare Life Sciences) in phosphate buffered saline, pH 7.4. The Zika virus containing fractions in the flow through was optionally concentrated by diafiltration using either 100 kDa or 300 kDa cut off membranes. The concentrated virus fraction was used for virus inactivation. In an alternate method, the clarified viral harvest was inactivated with either BPL or formalin according the methods described in the succeeding sections and then loaded on the column. The purity of the virus was checked on 12.5% SDS-PAGE. There was no significant difference in the yield or in purity in inactivating the virus before and after purification. The virus could also be purified using cellufine sulphate, DEAE-Sephadex CM-sephadex with salt gradient and by gel filtration on Sepharose CL-4B, ceramic hydroxyapatite column with gradient of 0.2M to 0.8M phosphate and in all cases followed by diafiltration using 100 or 300 kDa cut off membranes. The purity of the virus preparation was checked by silver staining of the virus sample in 12.5% of SDS-PAGE gel (See Figure 1).Zika virus by the aforementioned methods could be purified to high purity suitable to be used as vaccine bulk antigen. The virus could also be purified by ultracentrifugation on a 20-60% sucrose gradient using P28S rotor in Hitachi HIMACultracentrifuge after centrifugation at 100,000 x g for 6 to 8 hours.

Example 3: Zika Virus inactivation

Zika virus sample was inactivated (killed) by various methods for use as vaccine antigens. Formalin inactivation was tested at various concentrations ranging from 1:1000 (formalin: virus, v/v) to 1: 4000 (formalin: virus, v/v) at temperature 25±5^{0}C, more specifically at 22^{0}C and the kinetics of virus inactivation was monitored every 24 hours for up to 10 days, and routinely the virus inactivation was carried out at 25 ± 3^{0}C, preferably at 22^{0}C for 7 days. The virus inactivation was effective at all concentrations from 1:1000 v/v formalin: virus, up to 1:3500 v/v formalin: virus, at the aforementioned temperatures and time intervals. A ratio of 1:4000 v/v of formalin: virus was effective in virus inactivation at higher temperatures up to 30 to 37^{0}C for 3 to 7 days. Formalin inactivation was effective at all the aforementioned

ratios of formalin to virus at temperatures ranging from 2-8^{0}C when incubated for time intervals longer than 10 days. Hence formalin inactivation offers flexibility of virus inactivation at any temperature from 2^{0}Cto 37^{0}C at time intervals ranging from 24 hours to more than 10 days depending upon the conditions used for inactivation. Zika virus inactivation with Beta propiolactone (BPL) was tested under various conditions. Zika virus was completely inactivated at BPL concentrations ranging from 1:1000 (BPL: virus, v/v) up to 1: 3500 (BPL: virus, v/v) at temperatures from 25±5 ^{0}C for 24 to 48 hours. At higher concentration of BPL or at higher temperatures up to 37^{0}C, complete inactivation was achieved in 24 hours or less, and can be used as a method for quick inactivation of the virus. Zika virus could also be inactivated at the aforementioned concentrations of BPL when incubated at 2 to 8^{0}C for 3 to 7 days. A combination of BPL inactivation at 1:3500 (BPL: virus, v/v) at 22-25^{0}C for 48 hours, followed by treatment with low concentrations of formalin from 1:3000 to 1: 4000 v/v of formalin: virus for 24 hours was effective in both inactivating and stabilizing the virus. Any concentration of BPL and formalin could be used for both inactivation and stabilizing the virus, as long as inactivation is complete without deleterious effect on immunogenicity. Inactivation was tested from 0.005% up to 3% final concentration of Hydrogen peroxide at 20^{0}C to 25^{0}C for a period 2 hours. There was no deleterious effect on the immunogenicity of the virus at lower concentrations of hydrogen peroxide with very brief exposure times within minutes but was deleterious at prolonged concentrations at higher dose ranges tested. The inactivated virus samples after exposure to different time and dose concentrations were titered for infectious virus particles if any, by TCID50/mL from 5 minutes up to 6 hours at intervals of 5, 10, 20, 30 and 60 minutes and at 2, 4 and 6 hours. At higher concentrations, the virus was inactivated within seconds. At each time point, the reaction was stopped by addition of 10U/mL of catalase that rapidly hydrolyses hydrogen peroxide. The optimum concentration for inactivation was 0.01% final for duration of 60 minutes or less as determined by titration for infectious particles by TCID50/mL and subsequent immunogenicity. Zika virus inactivation with hydrogen peroxide offers the flexibility of duration of exposure at different concentrations for different time points according to the concentration of virus particles in the sample.

The purified Zika virus sample was heat inactivated at temperatures 50^{0}C to 65^{0}C for up to 60 min. UV inactivation of the virus was carried out UV exposure at 254 nm for up to 120 minutes.

Zika virus was inactivated by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy from a ^{60}Co source at the Gamma Agro Medical Processing Facility at Hyderabad. All the above inactivation methods were carried out in the presence and absence of virus stabilizing agents such as various concentrations of sugars such as sucrose, lactose, trehalose, maltose, mannose among others. The sugar alcohols used for conferring stabilizing effect were sorbitol and mannitol. The amino acids tested were selected from L-Histidine, L-Glutamic acid, L-Glycine and L-Aspartic acid and L-Glutamine and also human serum albumin and a combination of one or more of the aforementioned stabilizing agents. The most effective stabilizing agents were sorbitol at 0.5% to 2%, preferably 1.0% in combination with L-Glycine from 0.5% to 2%, preferably at 0.5%. Mannitol and L-glycine in combination was effective in stabilizing the virus sample during inactivation rather than Mannitol and L-glycine alone.

The Zika virus samples inactivated by all the aforementioned methods for use vaccine antigens were tested for completeness of inactivation by serially passaging the inactivated samples three times serially in Vero cells and testing for infectious virus at the end of

inactivation period by $TCID_{50}$. In addition to that, the inactivated virus sample after three serial passages *in vitro* was injected intracranially in 2-day old mice and observed for mortality or growth abnormalities for 21 days and considered completely inactivated when it showed no adverse effects *in vitro* and *in vivo* testing. No infectivity was observed with the formalin and beta-propiolactone inactivated virions at the aforementioned range of concentrations and for the various time periods tested. The inactivation kinetics of Zika virus by formalin and BPL as a representative example of one of the methods disclosed above is provided in Figure 2 (Fig.2A and Fig. 2B)

Example 4:Recombinant cloning and Expression of Zika virus pRME protein

Synthetic gene of the nucleotide sequence SEQ ID NO.1 encoding the Open Reading Frame (ORF) of the prME protein of SEQ ID NO.3 of Zika virus was synthesized at GenScript, NJ, USA. The gene was PCR amplified with the primers listed below to obtain a ~ 2.1 kb fragment of SEQ ID NO.1 encoding the prME protein of SEQ ID NO.3. See Figure 3A.

FVFP: 5'AACTGCTCGAGGAATTCGGATCCAAC 3'
FVRP: 5' AATGGGCATGCCTGCAGGCGGCCGCTC 3'

The PCR amplified fragments was digested with EcoR1 and Not1 restriction enzymes and cloned into the EcoR1 and Not1 sites of the pFastBac plasmid vector (Life Technologies, Carlsbad, CA, USA) under the control of the polyhedron promoter by the methods described in the User's manual of Bac to Bac Baculovirus expression system ("An efficient site-specific transposition system to generate baculovirus for high-level expression of recombinant proteins, Life Technologies, USA). In brief, the method utilizes a site specific transposition of the expression cassette such as the recombinant pFastBac vector with the cloned inserts as described above into a baculovirus shuttle vector (bacmid) propagated in *E.coli*. Recombinant pFastBac vector containing one of the inserts SEQ ID NO.1 or SEQ ID NO.2 cloned under the control of the polyhedron promoter is transformed into competent cells of *E.coli* Max Efficiency DH10Bac™, that contains a baculovirus shuttle vector (bMON14272) and a helper plasmid (pMON7124) that facilitates transposition to allow efficient re-generation of the recombinant bacmid The recombinant bacmids were selected on ampicillin, gentamicin and kanamycin containing plates by blue/white selection using bluo-gal or X-gal, and IPTG. The recombinant bacmids after confirmation by PCR for the presence of the gene inserts was isolated by standard protocols described in the aforementioned User manual. About 1 µg of the bacmid DNA was used for transfection with Lipofectamine in *Spodoptera frugiperda* Sf9 insect cells (Life Technologies, Carlsbad, USA) grown in serum free insect cell medium. The methods used for transfection, isolation and titration of P1 viral stocks are exactly as described in the User's manual of Bac-to-Bac Baculovirus Expression system as given above. The P1 stocks were serially amplified twice to obtain high titer P3 stocks for expression of the recombinant prME proteins in Sf9 cells. High titer baculovirus stocks for expression of the prME protein of SEQ ID No.3 was expressed in 25 mL suspension culture of Sf9 cells and was further scaled up systematically up to 125 mL per 500 mL flask. Baculovirus infected cells from multiple flasks were harvested at 72 hours post-infection, pooled, washed once with 1 x

PBS, pH 7.6 and lysed in cell lysis buffer containing 10 mM phosphate, pH 7.6 with 50 mM NaCl, 1 mM PMSF and 5 mM EDTA. The cell lysate was centrifuged at 20,000 rpm for 30 minutes to remove the cell debris and the supernatant was concentrated using protein concentrators with 10 kDa cut off membrane. The concentrated sample was layered on pre-equilibrated 20% to 60% sucrose gradient and centrifuged at 100,000 x g for 6-8 hours.

Fractions containing the recombinant Membrane and Envelope protein was isolated and confirmed by Western blot (**Fig.3B**) using the rabbit MR766 polyclonal antisera. The purified recombinant protein is of the sequence of the contemporary Asian genotype of Zika virus expressed using the gene sequence SEQ ID NO.1, encoded the protein of SEQ ID No.3. The recombinant ME protein cross reacted with MR766 antibodies in Western blot and in ELISA and was formulated as vaccine antigen for testing in Balb/c mice as described in sections below.

Example 5: Vaccine Formulations

Zika virus vaccine antigen of any of the aforementioned methods in the preceding Examples was tested for immunogenicity in laboratory animals with and without adjuvants. High binding (> 95%) was observed to aluminum hydroxide (Alhydrogel® 2%,Brenntag) as the adjuvant, used at the dose range of 0.1 mg to 1.5 mg of aluminum (provided as aluminum hydroxide) per dose even when tested at the high antigen dose of 40 mcg. Binding was complete at all the concentrations of Zika virus antigens as well as vaccine combinations with CHIKV and JE antigens discussed in the succeeding sections that were used for testing in mice. Binding to aluminum hydroxide was carried out for three hours at ambient temperature. An aliquot of the formulation was centrifuged at 5000 x g for 5 min and the supernatant was tested for completeness of binding by antigen ELISA. The binding of the antigen was complete as it could not be detected in the supernatant by ELISA. The buffer for the adjuvanted formulations was 10 mM phosphate buffer, containing 154 mM NaCl, pH 7.40±0.2 and optionally containing 1% sorbitol and 0.5% L-Glycine. Other buffers used for specific formulations are mentioned below. The adjuvants listed below were tested for comparative immunogenicity and in all cases concentrations are provided per dose of the vaccine. Inactivated Zika virus antigen was tested at 10 µg per dose:

a) Inulin (Orafti-HPX, Beneo) was tested at 0.5 mg per dose; gamma inulin was prepared by the methods described in (Cooper and Steele, 1988)
b) A combination of aluminum hydroxide and inulin. A combination of inulin and aluminum hydroxide, algammulin was prepared at a ratio of 10:1 (10mg /mL inulin: 1 mg/mL aluminum as aluminum hydroxide) was tested at 0.5 mg per dose
c) Muramyl di peptide (L18-MDP) (tlrl-lmdp, Invivogen) at 10 µg per dose
d) MPL (lipid A, monophosphoryl from *Salmonella enterica*, L-6895-1 MG, Sigma Aldrich) at 25 µg per dose
e) Combination of 0.25 mg aluminum (as aluminum hydroxide) and 25 µg of MPL per dose
f) Oil in water emulsion (OWEM1) containing 9.75 mg of squalene (S3626-100ML, Sigma Aldrich), 11.86 mg of alpha-tocopherol (T3251-5G, Sigma Aldrich), 4.58 mg of Tween-80 (61771205001730, Merck) in 10 mM phosphate buffer, pH 7.4±0.2.
g) Oil in water emulsion 3 (OWEM2) containing 9.75 mg squalene, 1.175 mg of tween-80, 1.175 mg Span-85 (S7135-250ML, Sigma Aldrich) in 10 mM citrate buffer, pH 7.0
h) Poly IC (polyinosinic polycytidylic acid, potassium salt, Cat. NO. P9582-5MG, Sigma Aldrich)at 25 µg per dose
i) Cholecalciferol (Arachitol, Abbot) at 0.75 mg per dose
j) Resiquimod (SML0196-10MG, Sigma Aldrich) + Poly IC, 25 µg each
k) Resiquimod (25 µg) + Oil in water emulsion 2 containing 9.75 mg squalene, 1.175 mg of tween-80, 1.175 mg Span-85 (S7135-250ML, Sigma Aldrich) in 10 mM citrate buffer, pH 7.0

l) Aluminum 0.25 mg and 0.5 mg per dose provided as aluminum hydroxide

All the above formulations elicited high level of neutralizing antibodies and the results are depicted in **Figure 4.** The individual components of the aforementioned adjuvants and any of their analogues, derivatives, side chain substitutions and any modifications of any of the above components at varying concentrations can be used as non-toxic vaccine adjuvant components as long as they have immunopotentiating effect. Formalin inactivated and recombinant Zika vaccine antigens as described in the aforementioned sections each at a concentration of 10 µg per dose was lyophilized in combination with either of the following excipients: 1% mannitol and 0.5% Glycine, 5% sucrose and 1% trehalose, 5% sucrose and 1% maltose and 2% mannitol and 0.5% Glycine. The dry lyophilized formulation could be easily reconstituted in aqueous solution with water, normal saline and 10 mM phosphate buffered saline, pH 7.4±0.2. The stability of the formulation was tested at 37^0C for two weeks. No change in the cake characteristics was observed indicating the stability of the formulations. The moisture content was below 1%.

Example 6: Effect of stabilizing agents

The stability of the formalin inactivated vaccine bulk for use as non-adjuvanted vaccine antigen was tested for stability with the following concentration of stabilizing agents: a) 2% sorbitol and 1% L-glycine; b) 1% sorbitol and 0.5 % L-glycine c) 1% mannitol and 0.5% L-glycine; d) 1% mannitol and 0.5% L-glutamic acid e) 1% sorbitol and 0.5% L-glycine, 1% human serum albumin. Stability testing was done at 37^0C for 2 weeks and the antigen concentration was tested by ELISA before and after exposure at 37^0C. 1 µg and 10 µg of the non-adjuvanted formulation with 1% sorbitol and 0.5% L-Glycine was tested for immunogenicity in Balb/c mice as discussed in the succeeding sections.

Example 7: Potency testing of Vaccine formulations in animal models

Zika vaccine antigen inactivated by the aforementioned methods was tested in Balb/c mice in dose ranges from 0.125 µg up to 40 µg of antigen per dose with 0.25 mg aluminum per dose (as aluminum hydroxide) in a volume of 100 µL (injected in two sites at 50 µL per site) by intramuscular route on days 0, 14, 28. Initial testing on the effect of aluminum (provided as aluminum hydroxide showed that alum adsorbed vaccine gave higher titer of neutralizing antibodies than non-adjuvanted vaccine. About 1 and 10 µg of inactivated vaccine antigen without alum contained 1% sorbitol and 0.5% L-glycine as the excipients to confer stability to the vaccine antigens. Blood was drawn from retro-orbital sinus on days 13, 21 and 35 for estimation of neutralizing antibody titers by $PRNT_{50}$, total Ab titer by ELISA, Ab avidity and cytokine profiles. Blood withdrawal and testing after each dose gave data on the potency and safety of single, two doses and three doses of the vaccine preparations. The animals were each challenged on day 36 with 10e5 PFU of Zika virus by intravenous route. The blood samples were monitored for up to 7 days at 24 hour intervals for formalin groups and at two points at 48 hours and 96 hours for BPL inactivation groups for protection against viremia by $TCID_{50}$ (50% Tissue Culture Infectious Dose) and the virus titers if any, were expressed as $TCID_{50}$/mL. Animal challenge studies showed complete protection from viremia in 1 µg to 40 µg of the dose groups tested. Hence the BPL and formalin inactivated vaccine formulations were further tested at 0.5 µg, and at 0.25 µg 0.125 µg per dose by the IM route in Balb/c mice and were found to be immunogenic even at low dilutions. For the alum adjuvanted formulations, 0.25 mg of aluminum (as aluminum hydroxide) per dose was used as the placebo control and for non-adjuvanted formulations, 10 mM phosphate buffer containing 154 mM NaCl, 1% sorbitol and 0.5% L-Glycine, pH 7.40 was used as the vehicle

control. All the formalin and BPL inactivated formulations elicited high level of neutralizing antibodies and protected against viremia as depicted in **Fig.5A, Fig. 5B and Figure 6**. Antigen only formulations also elicited high level of neutralizing antibodies and was protected from virus challenge. Recombinant prME protein expressed in insect cells was formulated at two doses of 10 and 20 µg per dose with 0.25 mg aluminum (as aluminum hydroxide) per dose in Balb/c (8 nos) and injected intramuscularly at day 0 and day 21 elicited neutralizing antibodies and the data is provided in Table 1. Gamma irradiated and Hydrogen peroxide inactivated Zika virus antigen at dose concentration of 10 µg and formulated with 0.25 mg aluminium (as aluminium hydroxide) per dose was injected by IM route in Balb/c mice at day 0 and day 21 and blood was withdrawn on day 28 for estimation of neutralizing antibodies by PRNT50. Formalininactivated virus antigen at 10 µg was formulated with each of the adjuvants disclosed in Example 5 and was injected intramuscularly in 4-6 week old Balb/c mice (5 nos per dose group) and the blood was drawn at 21 days after vaccine administration for estimation of neutralizing antibodies and cytokines. Control groups was included for each of the adjuvants and no neutralizing antibodies ($\leq$ 10 by $PRNT_{50}$) could be detected and the data is not shown. Neutralizing antibody titers by $PRNT_{50}$ of the different adjuvanted formulations, used pooled sera from each group is presented in **Figure 4.** High level of neutralizing antibodies were elicited by the aforementioned adjuvanted formulations.

A combination vaccine of arbovirus antigens were prepared a the following concentrations and tested in Balb/c mice: a) 10 µg formalin inactivated Zika virus antigen, 20 µg of BPL inactivated Chikungunya virus antigen and 6 µg of formalin inactivated JE antigen in a trivalent vaccine combination b) 10 µg formalin inactivated Zika virus antigen and 20 µg of BPL inactivated CHIKV virus antigen c) 10 µg of formalin inactivated Zika virus antigen and 6 µg of JE virus antigen. All the above vaccine combinations were tested with 0.25 mg aluminum (as aluminum hydroxide) per dose in Balb/c mice (8 nos each) with appropriate controls that included either of the aforementioned antigens alone, and also control animals that received equivalent amount of alum. The animals were boosted at 14 and at 21 days after the first immunization. Blood was collected at 7 days after the last booster injection. The sera samples were used for estimation of neutralizing antibody by $PRNT_{50}$ for Zika, CHIKV and JEV. The buffer used in all the formulations was 10 mM phosphate buffer, pH 7.2 to 7.6 containing 154 mM NaCl. All the methods disclosed above are applicable to any genotype/genotypic variants/serotypes and strains of Chikungunya virus, Zika virus and Japanese encephalitis viruses. See Table 1 for the results.

Table 1: Neutralizing antibodies elicited by various antigenic formulations as disclosed in the Examples.

Test Groups	Neutralizing antibody titers as $Log10PRNT_{50}$		
	Zika a	CHIKV	JE
Recombinant Zika prME – 10 µg x 2 doses	2.8	-	-
Recombinant Zika prME – 20 µg x 2 doses	3.22	-	-
Hydrogen peroxide inactivated Zika antigen – 10 µg x 2 doses	2.6	-	-
Gamma irradiated Zika antigen 10 µg x 2 doses	2.71	-	-
Zika alum adsorbed – 10 µg x3 doses	3.06	-	-
Chikungunya alum adsorbed –	-	2.808	-

20 µg x 3 doses			
JE alum adsorbed – 6 µg x 3 doses	-	-	3.06
Zika (10 µg) + CHIKV (20 µg) x 3 doses	2.95	2.68	-
Zika (10 µg) + JE (6 µg) x 3 doses	2.80	-	3.28
Zika (10 µg) + CHIKV(20 µg) + JE (6 µg) x 3 doses	2.79	2.63	3.21

Table 1 Legend: Purified recombinant prME antigen of Zika virus and the hydrogen peroxide inactivated and gamma irradiated Zika virus antigens formulated with 0.25 mg of aluminum per dose elicited neutralizing antibodies in Balb/c mice. The titers are expressed as Log10PRNT50 values. The vaccine combinations elicited neutralizing antibodies when two or more antigens were administered in a single formulation, and no significant antigenic interference was observed between JE, Zika and CHIKV viruses

Example 8: Passive immunization studies

The proof of concept that neutralizing antibodies are important immune correlates of protection against Zika virus infection was demonstrated by single injection of rabbit polyclonal Zika antisera with known titer, About 200 µL of antisera diluted 1:1 with PBS was injected intraperitoneally in Balb/c mice and challenged 8-24 hours later with 10e5 PFU of Zika virus by intravenous route in a volume of 100 µL. Equal no. of control animals received PBS, pH 7.4 and received the virus injection as the test animals. Blood was collected at 24, 48, 72, 96 and 144 hours post virus challenge for detection of viremia in both the group of animals. Passive immunization offered complete protection against viremia and infectious virus could not be detected by TCID50. See Figure 7. Viremia was detected in the control animals that persisted up to 6 days after virus challenge. Zika antibodies could be used as a therapeutic to ameliorate, eradicate or prevent Zika virus infections.

Example 9: Assays for Neutralizing Antibody Titers

Animal sera from all the aforementioned vaccine testing in mice described in Example 7 which include all the monovalent Zika vaccines inactivated with different inactivating agents and formulated with different adjuvants, vaccine antisera from dose ranging studies as well combination vaccines with CHIKV and JEV described in the preceding sections were assayed for neutralizing antibodies by 50% Plaque Reduction Neutralization Test ($PRNT_{50}$) by standardized procedures. Briefly, one day prior to the assay, 6-well plates were seeded with 2.5×10^3 Vero cells (ATCC CCL-81) per well and the plates were incubated at 37°C in a 5% CO_2 incubator. To 4-fold dilutions of the sera samples in MEM containing equal volume of the standardized Zika virus strain (10^5 pfu/mL) was added and incubated at 37°C with 5% CO_2 for 90 min. The cells were washed twice with 1 x PBS pH 7.4 (10 mM phosphate with 150 mM NaCl) and 0.30 ml of each dilution of the serum-virus mixture was added to the corresponding well and incubated for 90 min at 37°C in a 5% CO_2 incubator. Each assay was carried out in triplicates. The cells were overlaid with 2 ml of 0.85% methyl cellulose in MEM with 1% penicillin-streptomycin and 1% L-glutamine. The plates were incubated at 37°C in a 5% CO_2 incubator for 4 days. At the end of incubation, the plaques were fixed with 10% formalin, washed with 1 x PBS, pH 7.4 and were visualized with 0.1% crystal violet. The highest dilution of serum causing 50% reduction in the number of plaques formed by the control virus sample was estimated as the $PRNT_{50}$ titer. Anti-CHIKV and anti-JE antibodies from the vaccine combinations were also estimated PRNT50. All the aforementioned vaccine antigens elicited high level of neutralizing antibodies as depicted in **Figures 5 and 6**

Example 10: Zika virus cross neutralization studies

Formalin inactivated vaccine antisera cross neutralized the homologous MR766 virus strain of the African genotype and FSS13025 Zika virus strain (GenBank Acc No. JN860885) of the Asian genotype with EQUAL efficiency with PRNT50 titers of 18105 and 18325 against MR766 and FS13025 strains respectively. (The study BS-3018 was contracted to IBT Bioservices, Gaithersburg, MD, USA). Briefly, both the MR766 and the FS13025 Zika virus strains were diluted to ~ 250 PFU in serum-free medium. Both the vaccine antisera and control sera (placebo) were serially diluted in two-fold dilutions. The virus samples were mixed 1:1 with serially diluted sera samples and incubated at 37^0C for 2 hours. Vero cells seeded in 24-well plates were infected with the dilutions for 1 hour and 0.85% methyl cellulose was added to each well and incubated for 3 days. Cells were fixed and analyzed by plaque assay. The plates were scanned and the plaque counts were used to calculate the $PRNT_{50}$ titers using a 4PL curve fit. Hence the method of vaccine antigen preparation, formulation and testing are entirely applicable across any genotype of Zika virus as the vaccine with one genotype 100% cross neutralizes the heterologous strain and this also proves that no serotypes of Zika virus exists and that inactivated vaccine of Zika using any strain will be equally protective and potent as vaccine prepared using any genotype, and genotypic variant or indeed any Zika virus strain. This fact was further corroborated when the antibodies raised against the recombinant protein expressed as prME (protein of SEQ ID No.3) in insect cells cross neutralized the MR766 virus with high efficiency. The protein of SEQ ID No.3 is derived from the prME of the African genotype of Zika virus strain H/PF/013, which is the more contemporary strain of the Asian genotype. Cross neutralization of the vaccine antisera of the homologous MR766 strain of nucleotide SEQ ID NO.5 encoding the complete ORF of SEQ ID NO.6 and the heterologous FSS13025 of the SEQ ID No.7 encoding the complete ORF of SEQ ID NO. 8 is depicted in **Figure 8A and Fig. 8B.**

Example 11: Antibody ELISA

Briefly, Zika virus antigen was coated at the standardized concentration in coating buffer in 96-well plates overnight at 2 to 8°C. The plate contents were discarded and the wells were blocked with blocking buffer and washed extensively before adding the vaccine antisera at serial dilutions. Each vaccine antisera was assayed in triplicates. The plates were incubated for 90 min at 37°C, before adding secondary antibody (anti mouse-IgG HRPO conjugate) diluted 1:2500 in antibody diluent buffer. Each of the wells were washed five times with washing buffer (PBST, pH 7.4) and three times with PBS (pH 7.4), 30 seconds each. About 100 µl /well of freshly prepared substrate solution was added and incubated at ambient temperature for 10 minutes for color development. The color development was stopped by addition of 50 µL /well stop solution. Absorbance was read at 492 nm and the results recorded. For each assay, antigen blank, primary and secondary antibody blanks were included as controls. Seroconversion cut off value = pre-exposure average titer + (3 x standard deviation). The end point dilution of positively seroconverted sample which shows a titer equivalent to the pre-exposure level titer was identified. Reciprocal of the penultimate dilution of end point of a positively seroconverted sample was interpreted as the antibody endpoint titer. Antibody titers to both BPL inactivated and formalin inactivated Zika vaccine formulations were higher with aluminum hydroxide than with antigens alone. Vaccine formulations of the formalin inactivated vaccine at all doses (**Fig. 9A-9C**) and all doses of BPL inactivated vaccine (data not shown) elicited high level of antibodies, after each dose of vaccine administration confirming that vaccine can be administered as a single dose or two or more doses for eliciting a robust immune response against Zika virus.

Example 12: Antibody avidity

The quality of antibody responses to the vaccine was estimated by antibody avidity assays. The antigen-antibody binding avidities are the degree of affinity maturation in the B-cells. Higher antibody avidities correlate with neutralizing antibodies in several vaccine studies. Prior to determination of avidity index, titrations with sodium isothiocyanate (NaSCN) from 0 M to 6 M concentration in 0.25 M steps from 0 to 2.0 M were performed. After addition and incubation of primary antisera to the antigen coated plates, the plates were incubated with graded concentrations NaSCN for 15 min with intermittent shaking, washed and developed as in regular ELISA. The optical densities obtained at each of the concentrations were plotted. The highest OD (A) was plotted and halved (A/2), and the distance between the OD curves at A/2 was measured as the NaSCN shift value. The NaSCN shift was higher after the first booster dose compared to the prime dose and remained static or marginally increased further after second booster dose administration indicating that high affinity antibodies developed over time and with booster injections. A reference point in the ELISA titration was taken calculation of avidity index, (AI]) which is the ratio of antibody concentration (measured by absorbance) in ELISA of serum samples treated with and without the chaotropic agent NaSCN. Even at the lowest single dose concentration of 1 µg of formalin inactivated Zika vaccine, antibodies with high affinity binding to the antigen was detected, indicating the vaccine is potent even at low concentrations of the vaccine antigen (See **Figure 10**).

Example 13: Cytokine profiling

Both Th1 and Th2 cytokines were estimated in mice sera after administration of two doses of the formalin inactivated Zika antigen formulated with different adjuvants including aluminium hydroxide and in antigen only controls for comparison. The Mouse ELISA kit - Th1 / Th2 (Catalog No. 88-7711-44, eBioscience) was used for the estimation of IL-2, IFN gamma, IL-4 and IL-10 by methods exactly as per the kit protocols using the standards provided in the kit. The concentration of the cytokines are expressed in pg/mL. The results for Th1 cytokine levels are depicted in **Figure 11A and Fig.11B** and Th2 cytokines in **Fig12A and Fig.12B.**.

Example 14: Estimation of virus titers

The amount of infectious virus particles in the upstream and downstream bioprocess samples, Zika virus titers for animal challenge studies were measured by TCID50 (50% Tissue Culture Infectious Dose) assay. This assay measures the dilution of the virus sample that generates cytopathic effect (CPE) in 50% of the cells. Vero cells were seeded in 96-well microplates and incubated in 5% CO2 at 37°C overnight. The cells were infected with 10-fold serial dilutions of virus sample, followed by incubation for 5 d in 5% CO2 at 33°C. The cells were visually inspected for CPE and the TCID50 titer was calculated according to the method of Reed and Muench (Reference). The results are presented as a log10 titer (10xTCID50 units/mL). Alternatively plaque assays were used and the titers were expressed as plaque forming units, PFU /mL.

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We claim:

1. A stable vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, said antigens being formulated with or without an adjuvant in pharmaceutically acceptable buffer, wherein the vaccine composition elicits protective immune response to each of the viruses in mammals.

2. The vaccine composition as claimed in claim 1, wherein said Zika virus antigen is effective for treatment, diagnosis and prophylaxis against any genotype/genotypic variants/strains of Zika virus.

3. The vaccine composition as claimed in claim 2, wherein the composition is effective against any genotype/genotypic variants/strains/synthetic Zika viruses that share anywhere between 50% to 100% identity at the amino acid level in any region of the genome.

4. The vaccine composition as claimed in claim 3, comprising Zika virus antigens of any genotype/genotypic variant/strains/synthetic Zika virus, wherein the antibodies against any of the aforementioned Zika virus types cross neutralizes the homologous virus or any heterologous Zika virus strain that shares at least 50% -100% amino acid identity in any region of its whole genome, particularly the envelope E protein.

5. The vaccine composition as claimed in claim 1, wherein the antigens of Zika virus, Chikungunya virus and Japanese encephalitis virus are inactivated whole virion (virus) antigens.

6. The vaccine composition as claimed in claim 1, wherein Zika and Chikungunya virus antigens are purified recombinant antigens.

7. The vaccine composition as claimed in claim 1, wherein Zika virus antigen is prepared using Vero cells as cell substrate by adapting the virus to Vero cells.

8. The vaccine composition as claimed in claim 1, wherein said Zika virus antigen is a purified and concentrated antigen obtained from one or more methods selected from:
    a. ultracentrifugation;
    b. density gradient centrifugation;
    c. clarification of the viral harvest using membrane filtration, followed by purification by column chromatography; and
    d. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.

9. The vaccine composition as claimed in claim 8, wherein said purification by column chromatography comprises gel filtration, mixed mode resin column chromatography, ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography.

10. The vaccine composition as claimed in claim 9, wherein the column chromatography elutes majority of the virus antigen in the flow through such as Capto Core 700, most

preferably Capto Core 700 wherein the virus sample is purified on Capto Core 700 column and is eluted in the flow through.

11. The vaccine composition as claimed in claim 5, wherein the Zika virus is inactivated by at least one or more of a chemical inactivating agent, a physical inactivating agent and an irradiating agent.

12. The vaccine composition as claimed in claim 11, wherein the inactivation of Zika virus is carried out before or after purification of the virus.

13. The vaccine composition as claimed in claim 12, wherein the Zika virus is inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.

14. The vaccine composition as claimed in claim 13,wherein the Zika virus is inactivated by any one of the following methods selected from:
    a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8^0C to 37^0C, preferably 25 ± 3^0C, for at least 1 to 7 days;
    b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2^0C to 8^0C for at least 10 to 30 days;
    c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs at temperatures ranging from 8^0C to 30^0C, preferably 25 ± 3^0C, for 48 hours;
    d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2^0C to 8^0C for at least 3-7 days;
    e. A combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL :virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15^0C to 30^0C, preferably 25 ± 3^0C ;
    f. Hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30^0C for 5 minutes to 120 minutes.

15. The vaccine composition as claimed in Claim 11, wherein the inactivation of the Zika virus by irradiating agent comprises inactivation by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ^{60}Co source.

16. The vaccine composition as claimed in claim 11, wherein inactivation of the Zika virus by irradiating agent comprises inactivation by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

17. The vaccine composition as claimed in claim 11, wherein the virus is inactivated by heat treatment at a temperature between 50°C to 65°C for 30 min up to 2 hrs.

18. The vaccine composition as claimed in claim 1, wherein the buffer is selected from the list comprising of phosphate buffer, citrate buffer, phosphate citrate buffer, borate buffer, tris(hydroxymethyl)aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents.

19. The vaccine composition as claimed in claim 18, wherein phosphate buffer is sodium phosphate buffer at concentration of 5 mM up to 200 mM of phosphate ions of any pH between 6.50 to pH 9, and optionally containing sodium chloride at a concentration of 50 to 200 mM.

20. The vaccine composition as claimed in claim 1, wherein the buffer maintains the pH in a liquid composition above pH 6.5, preferably above pH 7.0 throughout the bioprocess from viral culture up to preparation of purified inactivated virus bulk.

21. The vaccine composition as claimed in claim 11, wherein the inactivation of Zika virus is carried out in the presence of a stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof.

22. The vaccine composition as claimed in claim 21, wherein the stabilizing agent is selected from:
    a. 2% sorbitol and 1% L-glycine;
    b. 1% sorbitol and 0.5 % L-glycine;
    c. 1% mannitol and 0.5% L-glycine;
    d. 1% mannitol and 0.5% L-glutamic acid; and
    e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

23. The vaccine composition as claimed in 11, wherein the inactivation of Zika virus comprises inactivation of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.

24. The vaccine composition as claimed in claim 6, wherein the purified recombinant Zika virus comprises antigens of Zika virus comprising the envelope (E) protein, membrane (M) protein and optionally the non-structural 1 (NS1) protein as vaccine antigens for eliciting immune response for prophylaxis of Zika virus infections.
25. The vaccine composition as claimed in claim 24, wherein the Zika virus has the structural protein sequences as disclosed in SEQ. ID No. 3 and SEQ. ID No.4 corresponding to nucleotide sequences of SEQ ID. No. 1 and SEQ ID No.2 respectively, for use as vaccine antigens against Zika virus infections caused by genotypes or variants thereof.

26. The vaccine composition as claimed in claim 6, wherein the Recombinant DNA constructs comprises a (i) vector (ii) at least one nucleic acid fragment corresponding to SEQ ID NO.1 or SEQ ID NO. 2 encoding the amino acid sequence of the proteins of SEQ ID NO.3, SEQ ID NO.4 respectively which is applicable to any Zika virus protein sequences that share at least 70% amino acid identity to the aforementioned SEQ ID NO. 3 and SEQ ID NO.4.

27. The vaccine composition as claimed in claim 26 comprising a recombinant DNA construct, wherein the vector is an eukaryotic plasmid vector being cloned in a

eukaryotic host such as baculovirus for expression in insect cells as virus like particles (VLPs).

28. The vaccine composition as claimed in claim 24, wherein the recombinant protein of Zika virus is obtained by the process comprising the steps of:
    a. transfecting the recombinant plasmid DNA in insect cells;
    b. harvesting the cells and isolating the recombinant protein therefrom;
    c. purifying the protein by a method selected from ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation and fractionation with salt.

29. The vaccine composition as claimed in claim 1, wherein the structural antigens of Zika virus are expressed in any prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

30. The vaccine composition as claimed in claim 1, wherein the composition is obtained by a process wherein neutralizing antibodies are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

31. The vaccine composition as claimed in claim 1, further comprising an adjuvant.

32. The vaccine composition as claimed in claim 31 wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus.

33. The vaccine composition as claimed in claim 32, wherein the composition comprises aluminum hydroxide in a concentration range of 0.1 mg to 1.5 mg of aluminum per vaccine dose, preferably 0.25 mg to 0.5 mg aluminum per vaccine dose.

34. The vaccine composition as claimed in claim 32 wherein the adjuvant confers mucosal immunity and systemic immunity when administered in mammals.

35. The vaccine composition as claimed in claim 1, wherein the composition with Zika virus antigen is administered at any dose ranging from 0.125 µg to 100 µg per dose with or without an adjuvant, either as a single dose or in two or more doses to elicit an immune response in a mammal.

36. A method of eliciting a protective immune response in mammals including humans comprising administering the vaccine composition of claim 1 by any route comprising intramuscular, intradermal, subcutaneous, intravenous, oral, intranasal or transcutaneous routes.

37. A method of administering the vaccine composition of claim 1 by any method comprising needles and syringes including pre-filled syringes, microneedle patch, needle-free patch, inhalation and nasal sprays.

38. A method of *in vitro* or *in vivo* use of the Zika virus antibodies of the composition as claimed in claim 1 for preparation of immunodiagnostic and immunotherapeutic agents for Zika virus infections.

39. The vaccine composition as claimed in claim 1 comprising Zika virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses.

40. The vaccine composition as claimed in claim 39, wherein the Zika virus antigen and Japanese encephalitis virus inactivated antigens are present in the combination vaccine at concentrations ranging 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

41. The vaccine composition as claimed in claim 40, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus.

42. The vaccine composition as claimed in claim 41, wherein the adjuvant is aluminium hydroxide with 0.25 mg to 1.0 mg of aluminium content per vaccine dose.

43. The vaccine composition as claimed in claim 1 comprising Zika virus and Chikungunya virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses.

44. The vaccine composition as claimed in claim 43, wherein Zika and Chikungunya virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

45. The vaccine composition as claimed in claim 44, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which

is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus.

46. The vaccine composition as claimed in claim 45, wherein the adjuvant is aluminium hydroxide at 0.25 mg to 1.5 mg of aluminium content per vaccine dose.

47. The vaccine composition as claimed in claim 1comprising Zika virus, Chikungunya virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses.

48. The vaccine composition as claimed in Claim 47, wherein Zika virus, Chikungunya virus and Japanese encephalitis virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

49. The vaccine composition as claimed in claim 48, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantrioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus.

50. The vaccine composition as claimed in claim 49, wherein the adjuvant is aluminium hydroxide at 0.25 mg to 1.0 mg of aluminium content per vaccine dose.

51. The vaccine composition as claimed in claim 1, wherein the composition optionally comprises 2-phenoxyethanol preservative at a concentration of 2.5 to 5 mg/mL.

52. The vaccine composition as claimed in claim 1, when administered in a single dose or in two or more doses in mammals elicits both Th1 and Th2 immune response against any of the arbovirus antigens comprising Zika Virus, Chikungunya virus and Japanese Encephalitis virus and is suitable for administration to humans.

53. A method for preparation of a vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, the method comprising one or more steps of inactivation, producing recombinant protein, expressing structural antigens, purification and concentration of

the virus antigen wherein said purification and concentration of Zika virus comprises one or more steps selected from:

a. ultracentrifugation ;
b. density gradient centrifugation;
c. clarification of the viral harvest using membrane filtration;
d. purification by column chromatography;
e. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.

54. The method as claimed in claim 53, wherein the column chromatography method comprises gel filtration, mixed mode resin column chromatography, any ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography.

55. The method as claimed in claim 53, wherein the purification comprises purification by column chromatographic method that elutes majority of the virus antigen in the flow through such as Capto Core 700, most preferably Capto Core 700wherein the virus sample is purified on Capto Core 700 column and is eluted in the flow through.

56. The method as claimed in claim 53, wherein Zika virus is inactivated by one or more inactivating agents selected from a chemical inactivating agent, a physical inactivating agent and an irradiating agent.

57. The method as claimed in claim 53, wherein inactivation of Zika virus is carried out before or after purification of the virus.

58. The method as claimed in claim 57, wherein the Zika virus is inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.

59. The method as claimed in claim 56, wherein the Zika virus bulk is inactivated by any one of the following methods selected from:

a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8^0C to 37^0C, preferably 25 ± 3^0C, for at least 1 to 7 days;
b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2^0C to 8^0C for at least 10 to 30 days;
c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs, if not more, at temperatures ranging from 8^0C to 30^0C, preferably 25 ± 3^0C, for 48 hours;
d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2^0C to 8^0C for at least 3-7 days;
e. a combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL :virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15^0C to 30^0C, preferably 25 ± 3^0C ;
f. Hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30^0C for 5 minutes to 120 minutes.

60. The method as claimed in claim 56, wherein the virus is inactivated by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ^{60}Co source.

61. The method as claimed in claim 56, wherein the Zika virus is inactivated by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

62. The method as claimed in claim 56, wherein the Zika virus is inactivated by heat treatment from 50°C to 65°C for 30 min up to 2 hrs, preferably, 65°C for 1 hr.

63. The method as claimed in claim 56, wherein the inactivation is carried out in the presence of stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof.

64. The method as claimed in claim 63, wherein the stabilizing agent is selected from:
    a. 2% sorbitol and 1% L-glycine;
    b. 1% sorbitol and 0.5 % L-glycine;
    c. 1% mannitol and 0.5% L-glycine;
    d. 1% mannitol and 0.5% L-glutamic acid; and
    e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

65. The method as claimed in claim 56, wherein the inactivation methods are applicable to Zika virus of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.

66. The method as claimed in claim 53, wherein the method of producing the recombinant protein comprises the steps of:
    a. transfecting recombinant plasmid DNA in insect cells;
    b. harvesting the cells and isolating the recombinant protein therefrom;
    c. purifying the protein by at least one of the methods comprising of ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation, fractionation with salt.

67. The method as claimed in claim 53, wherein the method of expressing the structural antigens of Zika virus comprising expression system is any prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

68. The method as claimed in claim 53, wherein the method comprising neutralizing antibodies that are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

69. A vaccine composition as claimed in claim 1, wherein the composition is administered in a prime boost strategy, wherein the prime is the candidate inactivated vaccine and the boost is either the same vaccine or any other vaccine such as DNA vaccine, Chimeric Zika virus vaccine, virus like particles, deactivated Zika vaccine, live attenuated virus vaccine, recombinant subunit vaccine, vectored vaccine or any vaccine derived from synthetic Zika virus, wherein the neutralizing antibodies in each of them are elicited against Zika virus Envelope protein.

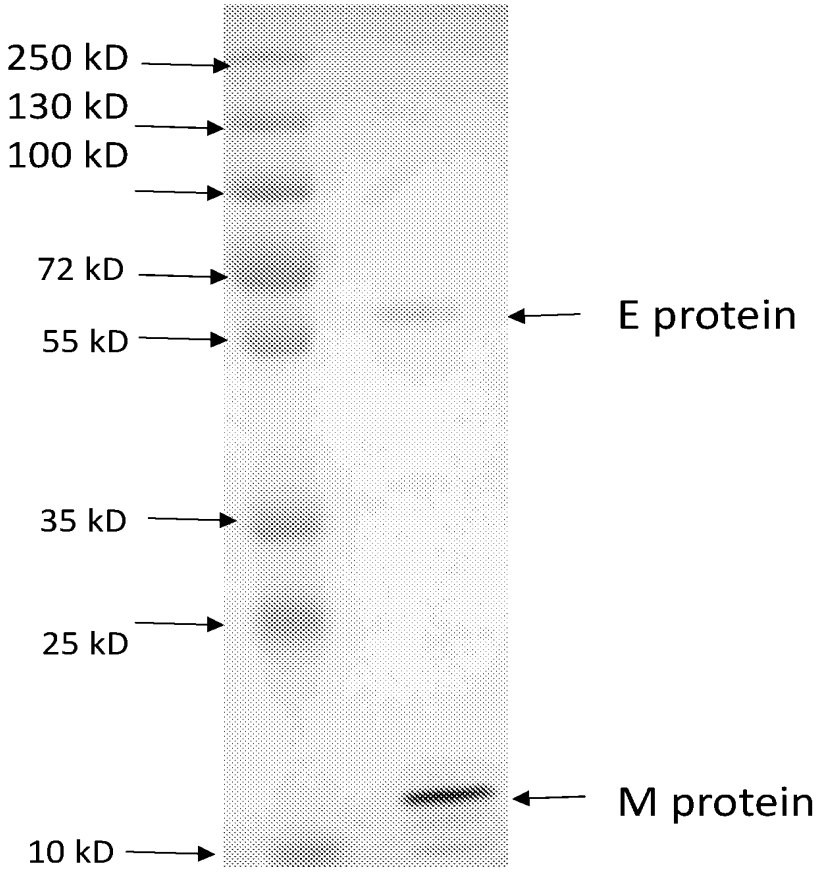

250 kD
130 kD
100 kD
72 kD
55 kD
35 kD
25 kD
10 kD
E protein
M protein

Figure 1

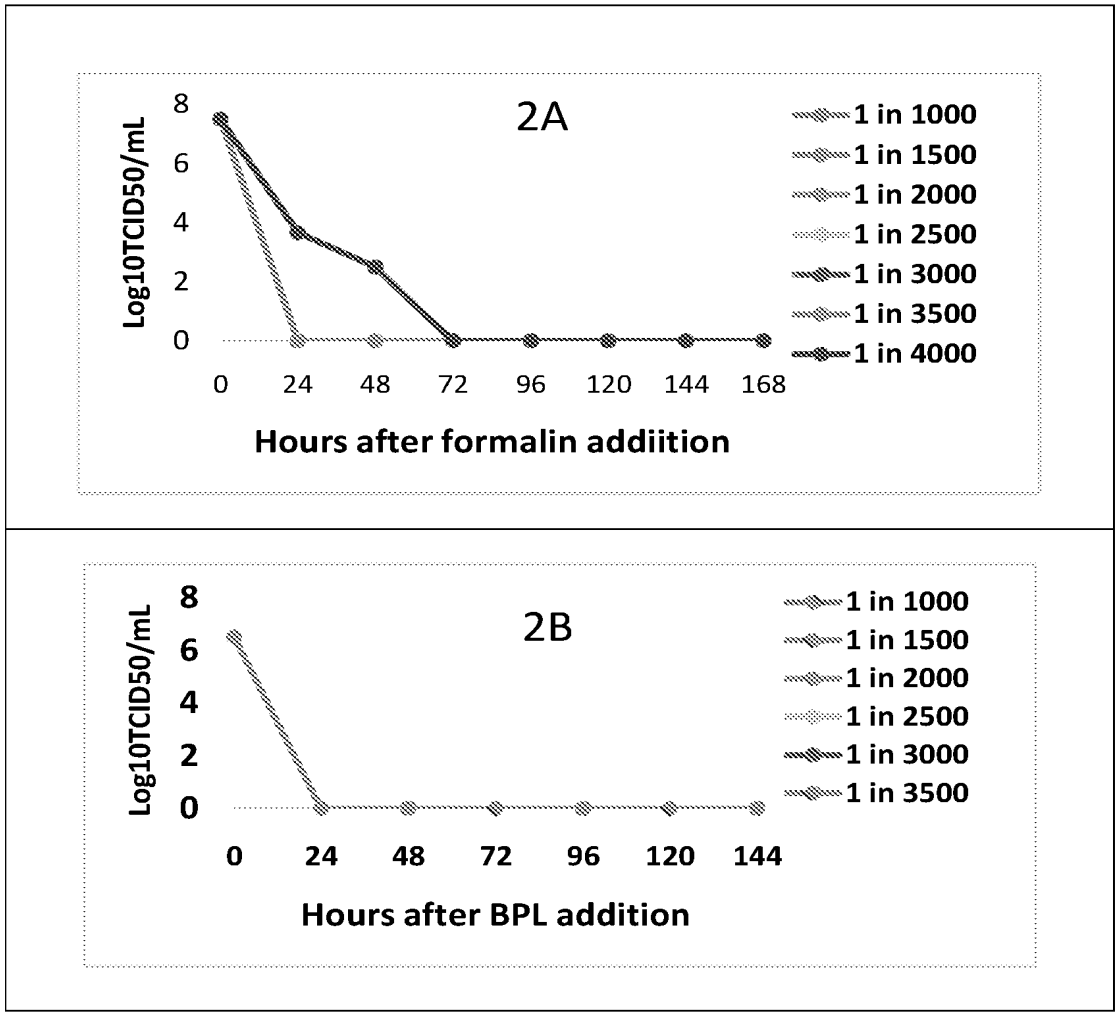

2A
Log10TCID50/mL
8
6
4
2
0
0
24
48
72
96
120
144
168
Hours after formalin addiition
1 in 1000
1 in 1500
1 in 2000
1 in 2500
1 in 3000
1 in 3500
1 in 4000
2B
Log10TCID50/mL
8
6
4
2
0
0
24
48
72
96
120
144
Hours after BPL addition
1 in 1000
1 in 1500
1 in 2000
1 in 2500
1 in 3000
1 in 3500

Figure 2

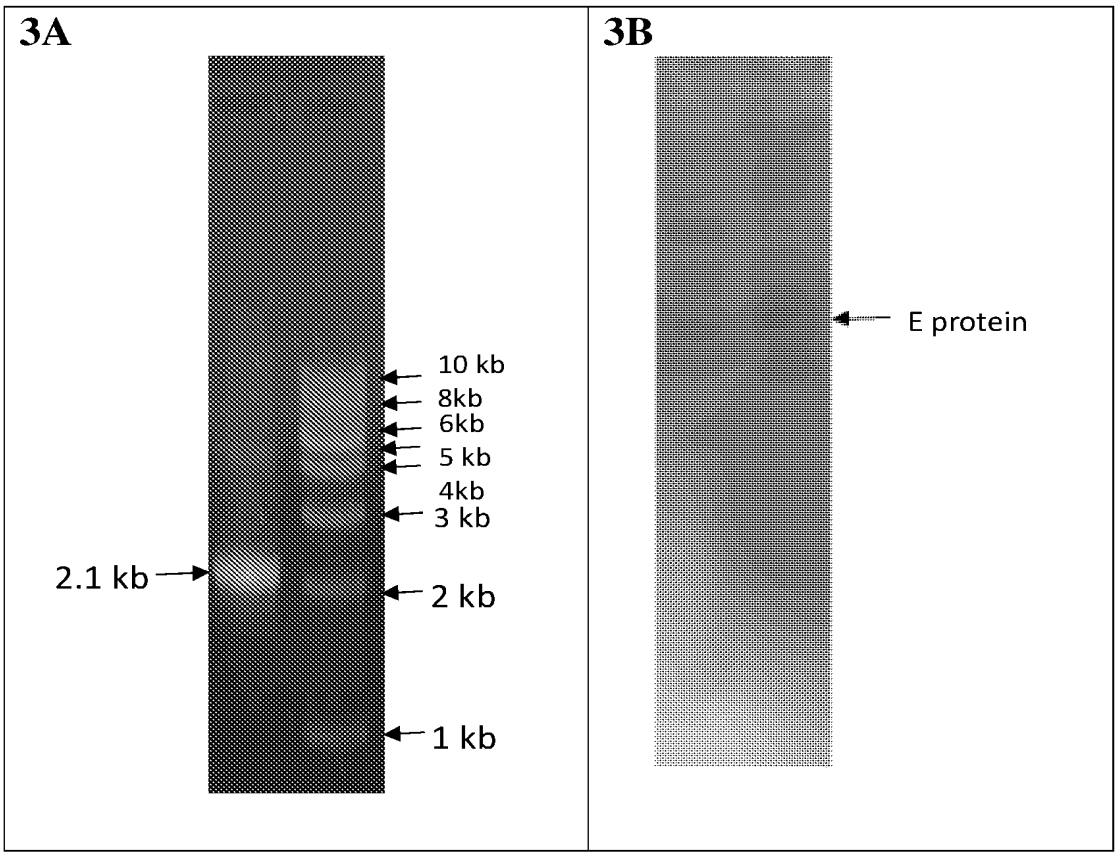


Figure 3

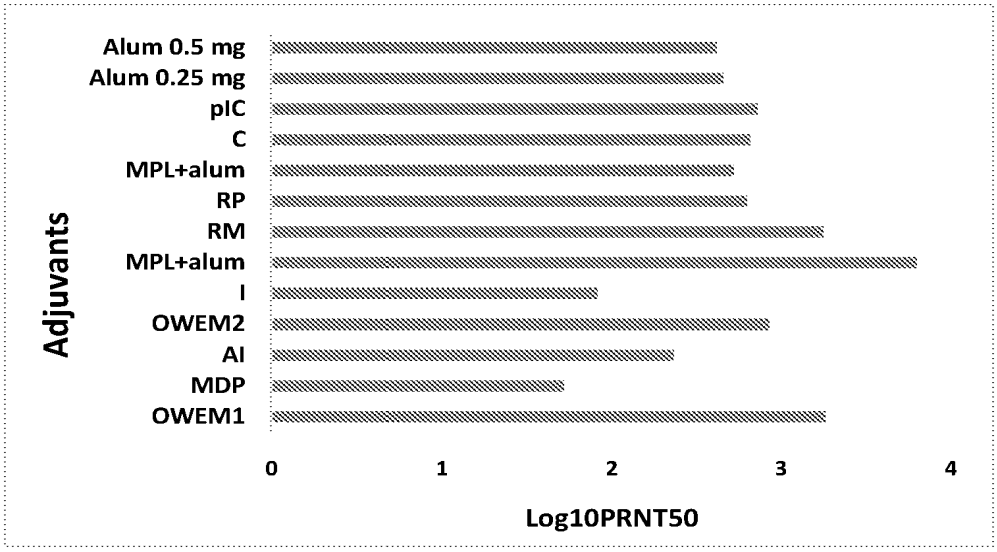
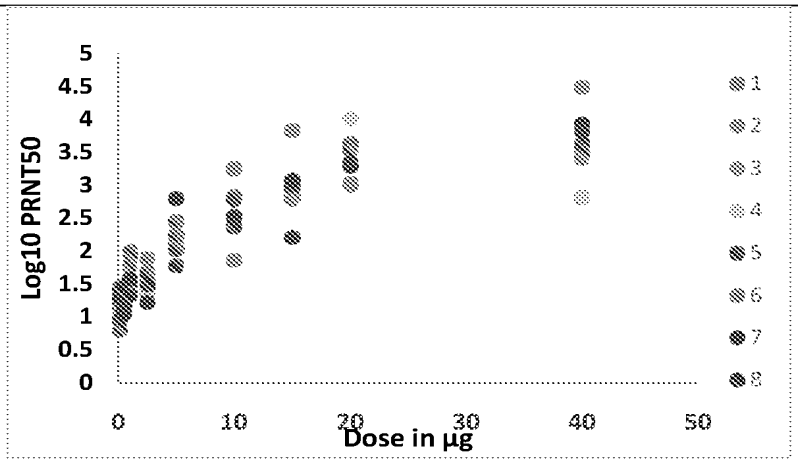
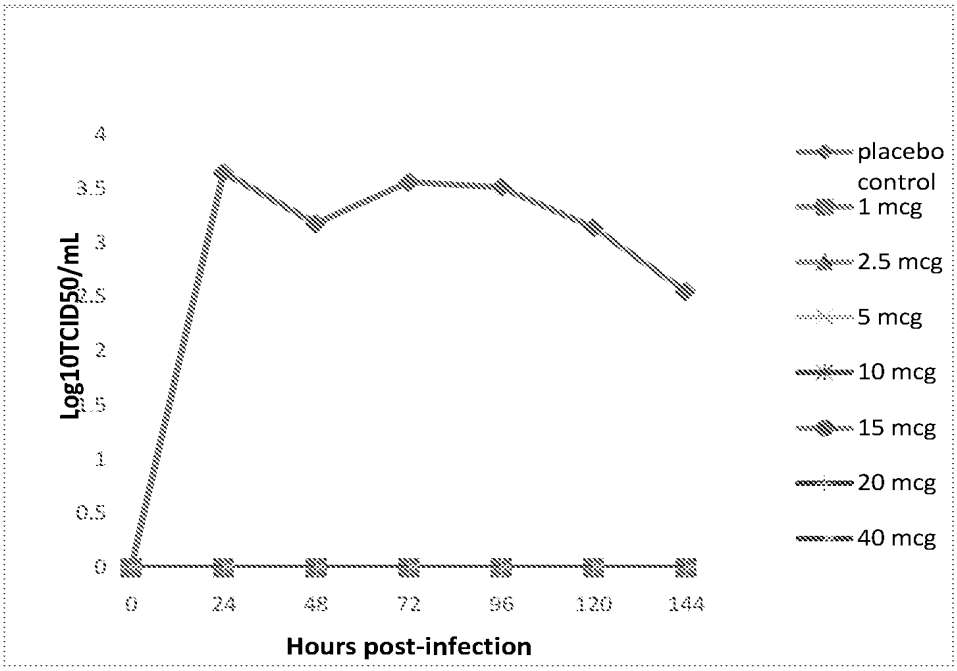

Alum 0.5 mg
Alum 0.25 mg
pIC
C
MPL+alum
RP
RM
MPL+alum
I
OWEM2
AI
MDP
OWEM1
Adjuvants
0
1
2
3
4
Log10PRNT50

Figure 4



5A



5B

Figure 5

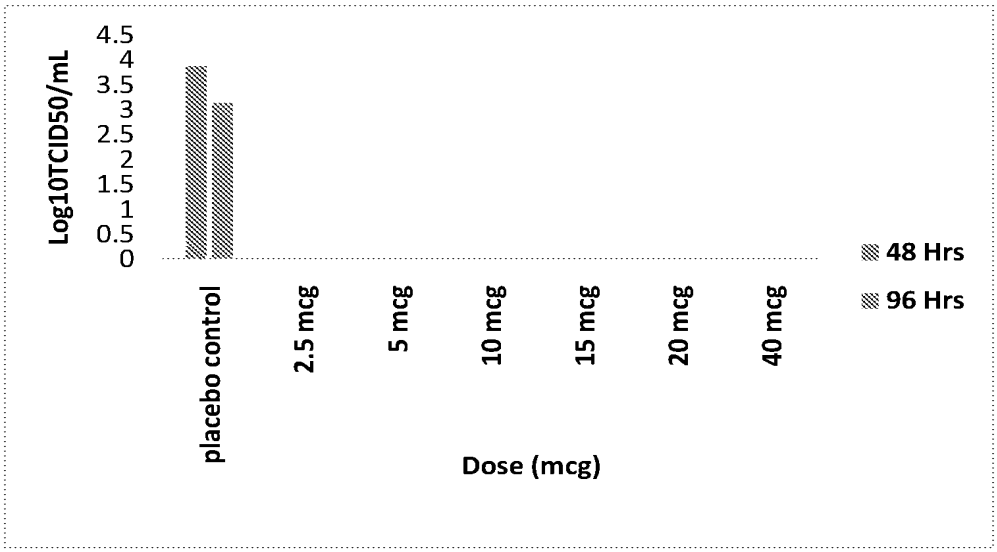

Log10TCID50/mL
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
placebo control
2.5 mcg
5 mcg
10 mcg
15 mcg
20 mcg
40 mcg
Dose (mcg)
48 Hrs
96 Hrs

Figure 6

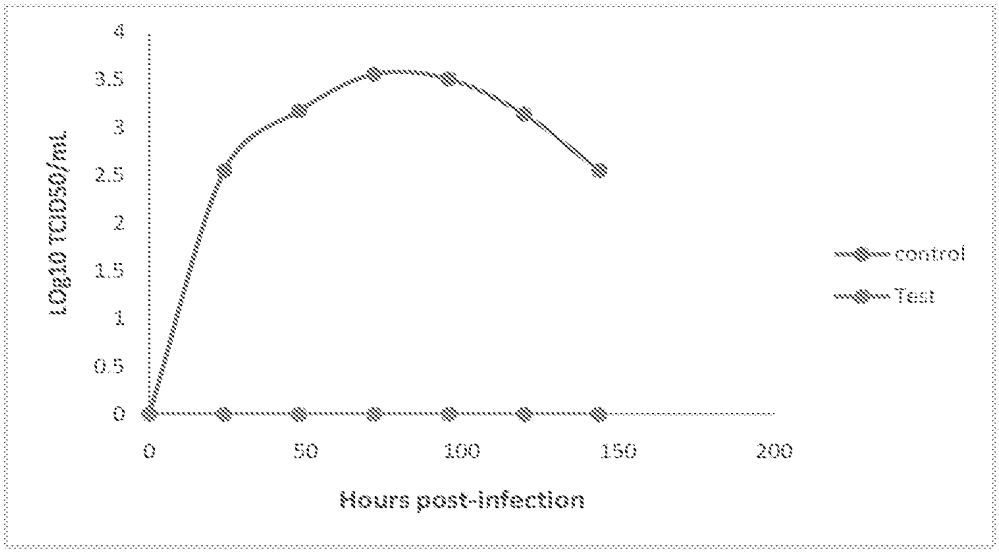

4
3.5
3
2.5
2
1.5
1
0.5
0
Log10 TCID50/ml
0
50
100
150
200
Hours post-infection
control
Test

Figure 7

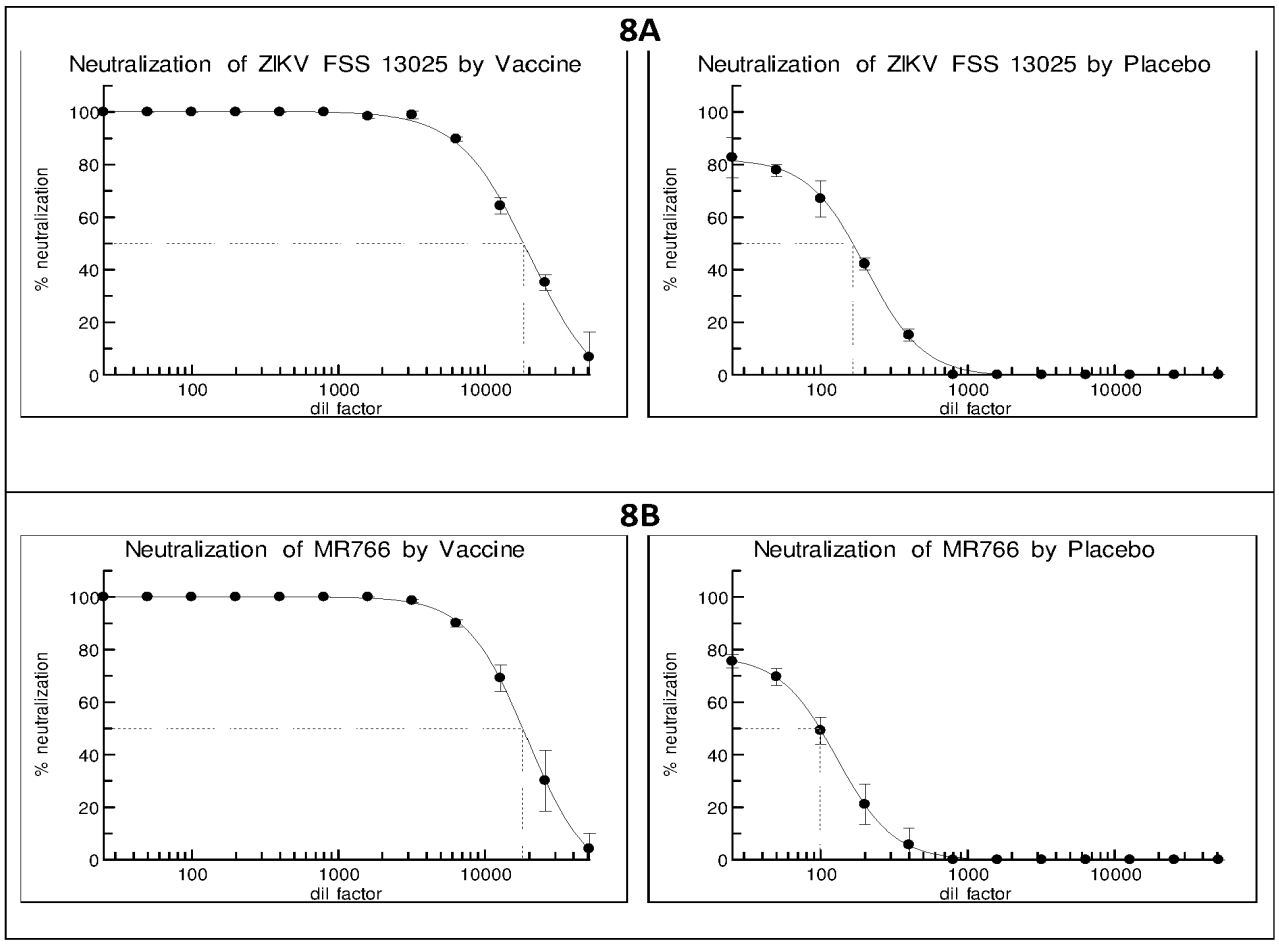

8A
Neutralization of ZIKV FSS 13025 by Vaccine
% neutralization
dil factor
Neutralization of ZIKV FSS 13025 by Placebo
% neutralization
dil factor
8B
Neutralization of MR766 by Vaccine
% neutralization
dil factor
Neutralization of MR766 by Placebo
% neutralization
dil factor

Figure 8

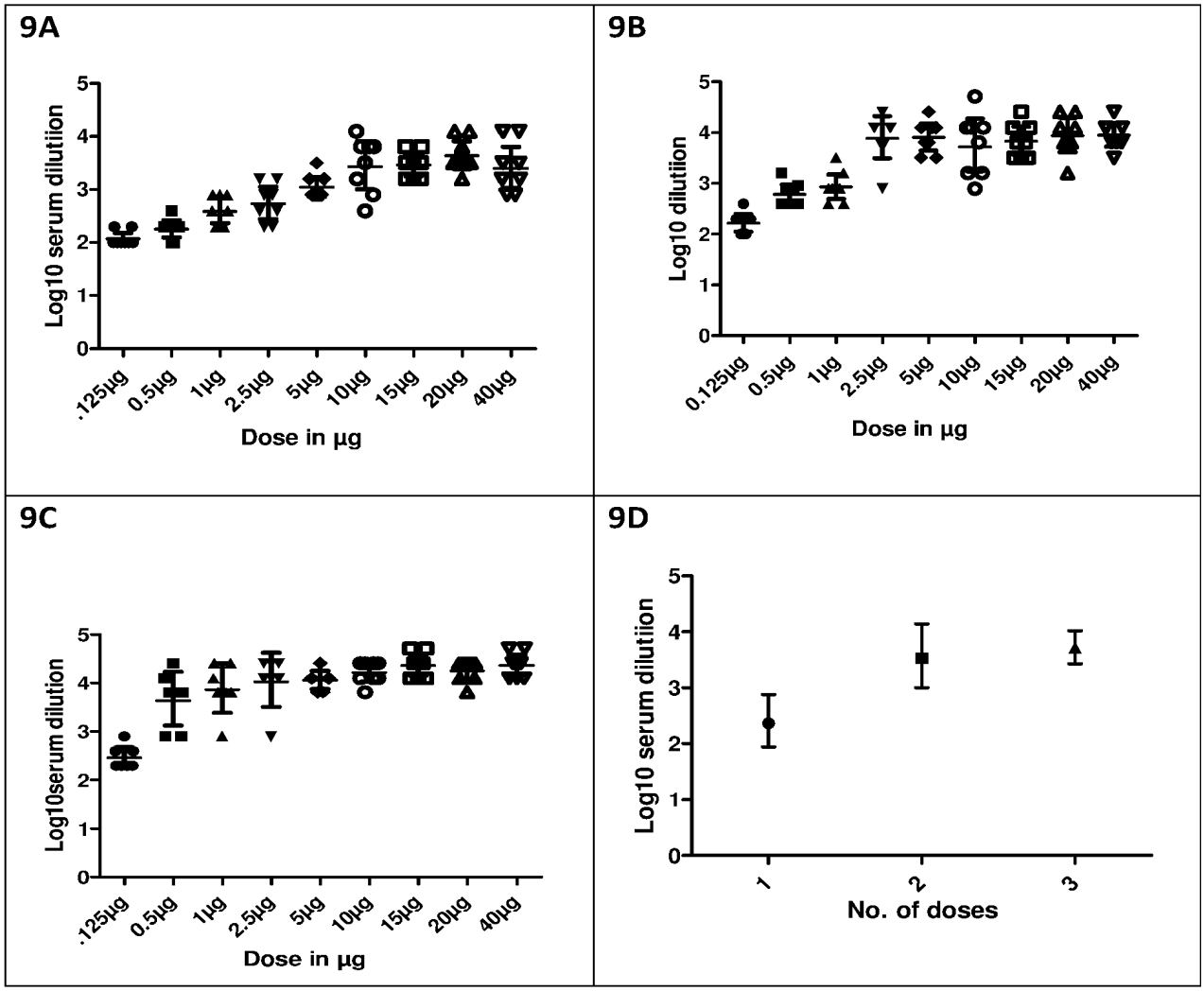

9A
Log10 serum dilutiion
Dose in µg
.125µg
0.5µg
1µg
2.5µg
5µg
10µg
15µg
20µg
40µg
9B
Log10 dilutiion
Dose in µg
0.125µg
0.5µg
1µg
2.5µg
5µg
10µg
15µg
20µg
40µg
9C
Log10serum dilution
Dose in µg
.125µg
0.5µg
1µg
2.5µg
5µg
10µg
15µg
20µg
40µg
9D
Log10 serum dilutiion
No. of doses
1
2
3

Figure 9


Avidity Index
4
3
2
1
0
1µg
2.5µg
5µg
10µg
15µg
20µg
40µg
Dose in µg

Figure 10

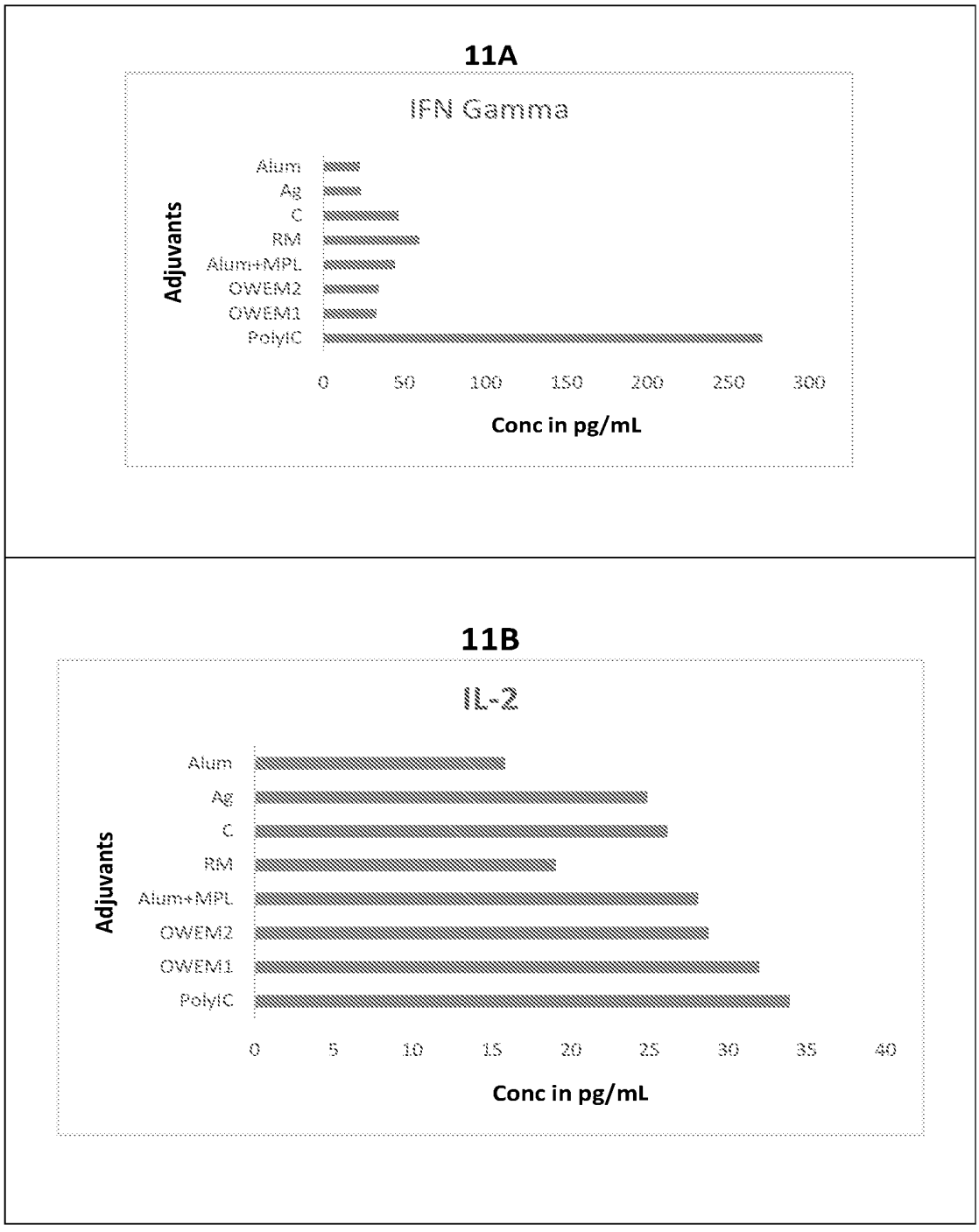

11A
IFN Gamma
Adjuvants
Alum
Ag
C
RM
Alum+MPL
OWEM2
OWEM1
PolyIC
0
50
100
150
200
250
300
Conc in pg/mL
11B
IL-2
Adjuvants
Alum
Ag
C
RM
Alum+MPL
OWEM2
OWEM1
PolyIC
0
5
10
15
20
25
30
35
40
Conc in pg/mL

Figure 11

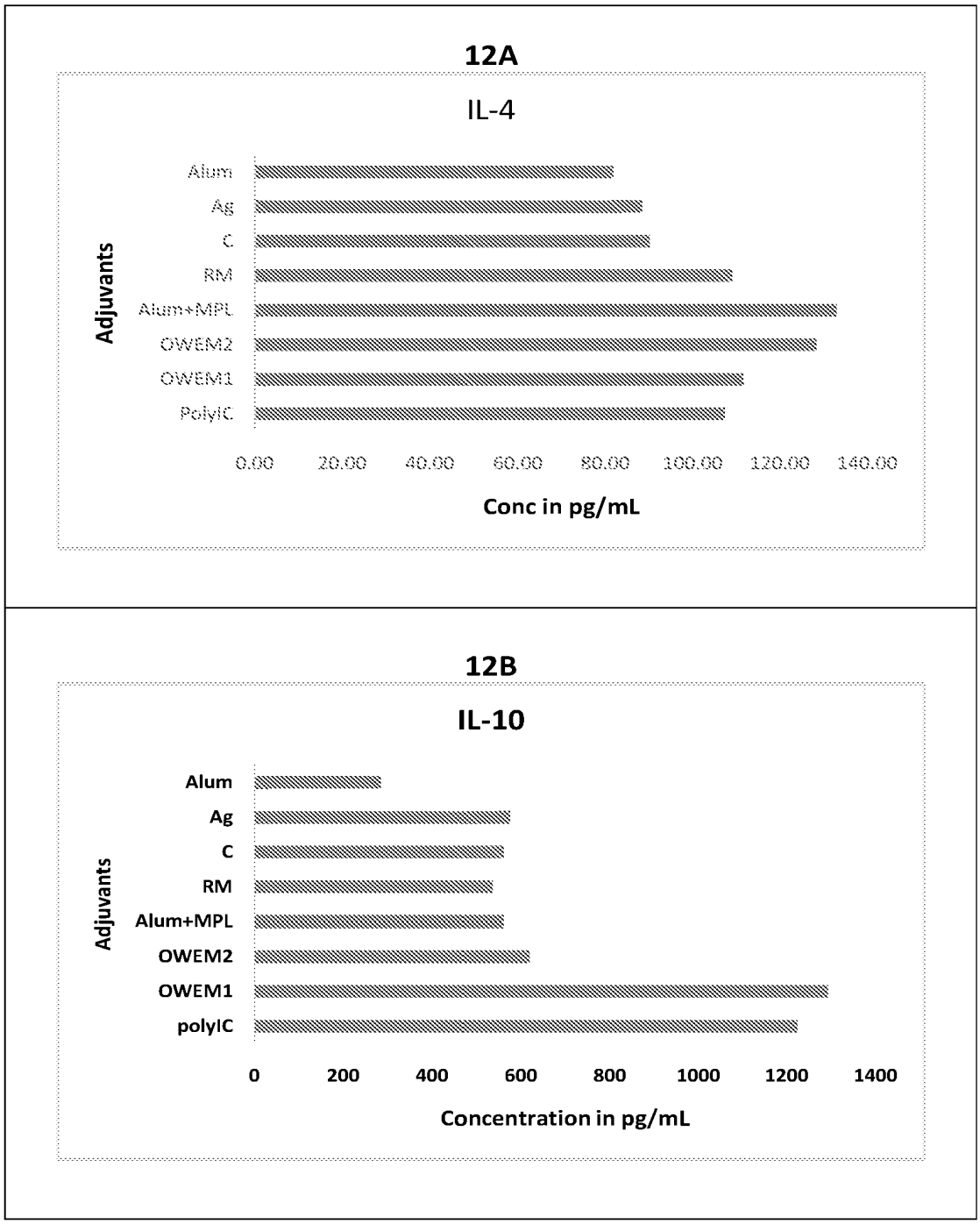

12A
IL-4
Adjuvants
Alum
Ag
C
RM
Alum+MPL
OWEM2
OWEM1
PolyIC
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
Conc in pg/mL
12B
IL-10
Adjuvants
Alum
Ag
C
RM
Alum+MPL
OWEM2
OWEM1
polyIC
0
200
400
600
800
1000
1200
1400
Concentration in pg/mL

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2016/050241

A. CLASSIFICATION OF SUBJECT MATTER
A61K39/12,C12N7/06 Version=2016.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K, C12N, A61K39/12, A61K39/39, A61P31/12, A61K38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Patseer, IPO Internal Database, NCBI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2008026225A2 (BHARAT BIOTECH INTERNATIONAL LIMITED) 06 Mar 2008; . . Examples 4, 8-9, 13-14; Claims	1, 5-9, 11-14, 17-20, 27-32, 35, 56-59, 62, 66-68
X	WO2012172574A1 (BHARAT BIOTECH INTERNATIONAL LIMITED) 20 Dec 2012; . . Examples 1-2, 6; Detailed description of the invention; Claims	1, 5-9, 11-22, 28, 31-33, 53-54, 56-64
X	WO2009082002A1 (THE KITASATO INSTITUTE) 02 July 2009; Claims	1,5

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14-12-2016	14-12-2016
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Anushri Kamble Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2016/050241

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 35-38,52, 69
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 35-38,52 and 69 relates to a method for treatment of the human or animal body by surgery or therapy, which does not require an international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

2. ☒ Claims Nos.: 2-4, 34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 2-4, 34 lack clarity as the claim attempts to define the invention solely by the result to be achieved, and no other technical
features are defined.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Citation	Pub.Date	Family	Pub.Date
WO 2008026225 A2	06-03-2008	IN 1583/CHE/2006 A	02-05-2008
		EP 2073839 B1	19-10-2016
		US 8865184 B2	21-10-2014
WO 2012172574 A1	20-12-2012	IN 2067/CHE/2011 A	21-06-2013
		EP 2720715 A1	23-04-2014
		JP 2014520117 A	21-08-2014
		US 2014120125 A1	01-05-2014
		AU 2012269907 A1	09-01-2014
WO 2009082002 A1	02-07-2009	IN 4638/DELNP/2010 A	24-02-2012
		US 2011020393 A1	27-09-2016
		EP 2233151 B1	11-05-2016
		JP 5580600 B2	27-08-2014

Espacenet

Bibliographic data: CN105749268 (A) — 2016-07-13

Inactivated Zika virus vaccine

Inventor(s): WANG LIN; LYU ZHE; HUI ZENGDI; GAO QIANG ± (王琳, ; 吕哲, ; 惠增弟, ; 高强)

Applicant(s): SINOVAC RES & DEV CO LTD ± (北京科兴中维生物技术有限公司)

Classification: **- international:*A61K39/12; A61P31/12***
- cooperative: A61K39/12; C12N2770/24034; Y02A50/392 more

Application number: CN20161222124 20160411 Global Dossier

Priority number (s): CN20161222124 20160411

Abstract of CN105749268 (A)

The invention provides an inactivated Zika virus vaccine. The inactivated Zika virus vaccine is obtained by: performing ultrafiltration and concentration on Zika virus liquid after inactivation, centrifuging the concentrated virus liquid by adopting a sucrose density zone, performing ion exchange and concentration sterilization on a centrifugal product to obtain a Zika virus vaccine stock solution, diluting the vaccine stock solution until the total protein content is not more than 20mu g/ml, and adding an adjuvant to obtain a vaccine semi-finished product. The method for preparing the vaccine provided by the invention is simple, convenient and easy to operate, the cost is saved, the produced vaccine is suitable for Asian people, a unit dose of the Zika virus liquid is high in immunogenicity, the content of hybrid protein is low, the side effect after injection is small, and the safety is high, so that the vaccine is suitable for vaccination of fertile women before pregnancy, can avoid newborn Brazil microcephaly caused by infection of Zika virus, and is significant in social value and market efficiency.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

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(43)申请公布日 2016.07.13

(21)申请号 201610222124.3

(22)申请日 2016.04.11

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代理人 王文君

(51)Int.Cl.
A61K 39/12(2006.01)
A61P 31/12(2006.01)

权利要求书1页 说明书9页 附图1页

(54)发明名称

一种灭活的寨卡病毒疫苗

(57)摘要

本发明提供了一种灭活的寨卡病毒疫苗，是将寨卡病毒液灭活后进行超滤浓缩，浓缩的病毒液采用蔗糖密度区带离心，离心产物进行离子交换、浓缩除菌得到寨卡病毒疫苗原液，疫苗原液稀释至总蛋白含量不超过20μg/ml，加入佐剂得到疫苗半成品。本发明制备该疫苗的方法简便易操作，成本节约，生产的疫苗适用于亚洲人种，单位剂量寨卡病毒液免疫原性高，杂蛋白含量低，注射后副作用小，安全性高，适用于育龄妇女孕前接种，避免感染寨卡病毒导致新生儿巴西小头症，社会价值和市场效益显著。

CN 105749268 A

1.一种灭活的寨卡病毒疫苗，其特征在于，寨卡病毒抗原含量为100-1600U/ml，且总蛋白含量低于20μg/ml。

2.如权利要求1所述的灭活的寨卡病毒疫苗，其特征在于，寨卡病毒抗原含量为800U/ml。

3.如权利要求1所述的灭活的寨卡病毒疫苗，其特征在于，通过以下步骤制备得到：

(1)利用为载体培养细胞技术培养Vero细胞，当细胞生长成致密单层时，接种寨卡病毒工作种子，培养；

(2)将病毒培养液澄清过滤后收获病毒液，灭活；

(3)灭活的病毒液超滤浓缩，浓缩后的病毒液采用蔗糖密度区带离心；

(4)离心产物超滤脱糖，得到脱糖液；将脱糖液进行离子交换，得到层析液；层析液进行浓缩、除菌即为原液；

(5)将原液稀释，加入佐剂，得到疫苗半成品。

4.如权利要求3所述疫苗，其特征在于，步骤(2)所述的病毒培养液为M199与DMEM/F12按照体积比1:1混合得到。

5.一种制备灭活的寨卡病毒疫苗的方法，其特征在于，包括以下步骤：

(1)利用为载体培养细胞技术培养Vero细胞，当细胞生长成致密单层时，接种寨卡病毒工作种子，培养；

(2)将病毒培养液澄清过滤后收获病毒液，灭活；

(3)灭活的病毒液超滤浓缩，浓缩后的病毒液采用蔗糖密度区带离心；

(4)离心产物超滤脱糖，得到脱糖液；将脱糖液进行离子交换，得到层析液；层析液进行浓缩、除菌即为原液；

(5)将原液稀释，加入佐剂，得到疫苗半成品。

6.如权利要求4所述的方法，其特征在于，步骤(1)当细胞生长成致密单层时，按照MOI为0.05-0.3接种寨卡病毒工作种子，培养温度为35-37℃，培养时间为72-96小时。

7.如权利要求4所述的方法，其特征在于，步骤(2)中灭活方法为单次收获液中加入终浓度为100-400μg/ml的甲醛，37℃，灭活4-7天。

8.如权利要求4所述的方法，其特征在于，步骤(3)病毒液浓缩倍数为10-100倍，膜包可截留分子质量为100-300KD。

9.如权利要求4-7任一所述的方法，其特征在于，步骤(3)的蔗糖密度区带离心方法为：依次从离心机边孔以50-100rpm依次泵入超离缓冲液，超滤浓缩后的病毒液，低浓度蔗糖溶液，最后以50-150rpm泵入高浓度蔗糖溶液直至中孔流出液体离心转速为28000-35000rpm，离心时间为：6-20小时，离心温度为2-8℃；

低浓度蔗糖溶液蔗糖质量百分比为25％-45％，高浓度蔗糖溶液蔗糖质量百分比为55％-60％，超离缓冲液为0.01-0.05M PBS，pH值为：6.0-7.5。

10.如权利要求4-7任一所述的方法，其特征在于，将原液稀释至200-3200U/ml，与佐剂按照1:1等体积混匀。

一种灭活的寨卡病毒疫苗

技术领域

[0001] 本发明涉及生物制药领域，具体地，涉及一种灭活的寨卡病毒疫苗。

背景技术

[0002] 寨卡病毒病(Zika Virus Disease)是由寨卡病毒(Zika Virus)引起并通过蚊媒传播的一种自限性急性传染性疾病。寨卡病毒于1947年首次在乌干达恒河猴体内被发现，1952年在乌干达和坦桑尼亚的人体中被分离。2007年以前，全球仅报告14例寨卡病毒病散发病例，2007年首次在太平洋岛国密克罗尼西亚的雅普岛发现寨卡病毒暴发疫情，其后发现寨卡病毒感染病例和暴发疫情的国家及地区有增加趋势。2015年5月，巴西报告首例寨卡病毒病病例，截至2016年1月底，巴西等24个美洲国家和地区均发现了本地感染病例。同时，欧洲、北美等地的多个国家发现输入性病例，台湾也报告1例来自泰国的输入性病例。

[0003] 寨卡病毒属黄病毒科(Flaviviridae)黄病毒属(Flavivirus)，呈球形，直径约为40-70nm，有包膜。基因组为单股正链RNA，长度约为10.8Kb，分为亚洲型和非洲型两个基因型，目前在南美地区流行的病毒为亚洲型。

[0004] 感染寨卡病毒的主要表现为发热(多为中低度发热)、皮疹(多为斑丘疹)，并可伴有非化脓性结膜炎、肌肉和关节痛、全身乏力以及头痛，少数患者可出现腹痛、恶心、腹泻、粘膜溃疡、皮肤瘙痒等。约80％的人为隐性感染，仅有20％的人出现上述临床症状，一般持续2-7天后自愈，重症和死亡病例少见。有与寨卡病毒感染相关的格林-巴利综合征(吉兰-巴雷综合征，Guillain-Barre Syndrome)病例的报道，但二者之间的因果关系尚未明确。小儿感染病例还可出现神经系统、眼部和听力等改变。孕妇感染寨卡病毒可能导致新生儿小头畸形甚至胎儿死亡。寨卡病毒疫情中，巴西等国新生儿小头畸形病例数显著增加，疫情的快速蔓延以及与小头畸形之间的因果关系，引起了国际社会的广泛关注。

[0005] 我国南方部分地区存在可传播寨卡病毒的媒介伊蚊，近年来与之传播方式相似的登革热输入性疫情持续增多，并在南方部分省份引起了较大规模的暴发疫情。随着与相关国家或地区人员往来的日益密切，我国存在寨卡病毒输入风险。特别是我国南方地区夏秋季节伊蚊密度较高，一旦有病例输入，不排除在局部地区发生本地传播扩散的可能。

[0006] 目前全球范围内尚无有效的治疗手段对寨卡病毒病进行治疗，即使通过防止蚊虫来预防寨卡病毒侵染，在实施过程中也存在极大难度，尤其随着夏季的到来，蚊虫密度增加，根本无法完全防范蚊虫叮咬，因此，通过疫苗进行预防成为未来消灭寨卡病毒的有效手段。

[0007] 2016年3月1日WHO发布寨卡风险通报，关于寨卡病毒传播和潜在并发症方面的风险提供暂行指南。虽然1952年已经在人类中首次发现了寨卡病毒，但很少有关于疫情的记录。自2015年来寨卡病毒主要在美洲暴发疫情，传播加剧，而且这一期间的小头症和吉兰-巴雷综合征等神经系统并发症比率也出现上升。自2015年来巴西出现数十例小头症新生儿，为了尽量减少疫情的扩大和对人群的危害，WHO提出应加强对寨卡病毒疫苗的研发。然而目前为止尚无寨卡疫苗问世，疫苗的免疫原性和稳定性有待研究。

[0008] 由于寨卡病毒在本次疫情中突然暴发，虽然自上世纪50年代就已经分离出寨卡病毒，但是鲜有文献描述寨卡病毒，其培养工艺、纯化工艺以及制剂工艺尚为空白。

发明内容

[0009] 为了能够最大程度地预防寨卡病毒侵染，同时更好的实现优生优育的目标，本发明的目的在于提供了一种灭活的寨卡病毒疫苗，适用于各个年龄阶段人群接种，尤其对于育龄妇女，以有效防范寨卡病毒的侵袭。

[0010] 本发明的另一目的在于提供一种制备灭活寨卡病毒疫苗的方法。

[0011] 本发明提供的灭活的寨卡病毒疫苗中寨卡病毒抗原含量为100-1600U/ml，且总蛋白含量低于20μg/ml。

[0012] 优选地本发明，寨卡病毒疫苗中寨卡病毒抗原含量为800U/ml。

[0013] 本发明的灭活的寨卡病毒疫苗通过以下步骤制备得到：

[0014] (1)利用为载体培养细胞技术培养Vero细胞，当细胞生长成致密单层时，接种寨卡病毒工作种子，培养，

[0015] (2)将培养液澄清过滤后收获病毒液，灭活；

[0016] (3)灭活的病毒液超滤浓缩，浓缩后的病毒液采用蔗糖密度区带离心；

[0017] (4)离心产物超滤脱糖，得到脱糖液；将脱糖液进行离子交换，得到层析液；层析液进行浓缩、除菌即为原液；

[0018] (5)将原液稀释，加入佐剂，得到疫苗半成品。

[0019] 本发明提供的寨卡病毒培养基为M199与DMEM/F12按照1:1等体积混合。

[0020] 另一方面，本发明提供了一种制备灭活的寨卡病毒疫苗的方法，包括以下步骤：

[0021] (1)利用为载体培养细胞技术培养Vero细胞，当细胞生长成致密单层时，接种寨卡病毒工作种子，培养；

[0022] (2)将培养液澄清过滤后收获病毒液，灭活；

[0023] (3)灭活的病毒液超滤浓缩，浓缩后的病毒液采用蔗糖密度区带离心；

[0024] (4)离心产物超滤脱糖，得到脱糖液；将脱糖液进行离子交换，得到层析液；层析液进行浓缩、除菌即为原液；

[0025] (5)将原液稀释，加入佐剂，得到疫苗半成品。

[0026] 其中，步骤(1)当细胞生长成致密单层时，按照MOI为0.05-0.3接种寨卡病毒工作种子，培养温度为35-37℃，培养时间为72-96小时，病毒的培养基为M199与DMEM/F12按照1:1等体积混合。

[0027] 本发明方法中，步骤(2)中灭活方法为单次收获液中加入终浓度为200μg/ml的甲醛，37℃，灭活4-7天。

[0028] 其中，步骤(3)病毒液浓缩倍数为10-100倍，膜包可截留分子质量为100-300KD。

[0029] 进一步地，步骤(3)的蔗糖密度区带离心方法为：依次从离心机边孔以50-100rpm依次泵入超离缓冲液，超滤浓缩后的病毒液，低浓度蔗糖溶液，最后以50-150rpm泵入高浓度蔗糖溶液直至中孔流出液体离心转速为28000-35000rpm，离心时间为：6-20小时，离心温度为2-8℃；

[0030] 优选地，离心转速为30000rpm，离心时间为：14小时，离心温度为4℃。

[0031] 低浓度蔗糖溶液蔗糖质量百分比为25%-45%，高浓度蔗糖溶液蔗糖质量百分比为55%-60%，超离缓冲液为0.01-0.05M PBS，pH值为：6.0-7.5。

[0032] 本发明方法中，步骤(5)佐剂为氢氧化铝佐剂，与稀释后的原液体积为1:1

[0033] 本发明方法中，步骤(5)是将原液稀释到200-3200U/ml，按照1:1体积比与铝佐剂混匀。

[0034] 本发明具备以下有益效果：

[0035] (1)本发明首次制备出有效防护寨卡病毒的疫苗，能够很大程度缓解本次寨卡病毒疫情，极大的减少病患，为优生优育提供了良好的保障，育龄妇女在孕前接种本疫苗能够保护孕期寨卡病毒的侵袭，避免巴西小头症等类似新生儿疾病的发生，从而减轻社会和家庭的负担。本发明采用的毒株为国内输入性寨卡病毒患者血液分离而来的，以病毒为抗原制得的疫苗适用于亚洲人种免疫。本发明为国内首次大规模培养寨卡病毒，并制备寨卡病毒疫苗。

[0036] (2)本发明使用的细胞为Vero细胞，该细胞具有生长迅速，能够形成致密单层的优势，其安全性得到国际广泛认证。使用Vero细胞可以实现多次收获的目的，能极大的减少操作步骤和降低成本。本发明采用的是微载体技术培养Vero细胞，经过研究人员的筛选，制备出适合寨卡病毒生产的培养基，从而更加简便快速的培养出高数量级高密度适于接种病毒的细胞，节约生产成本，提高生产效率，适合于产品的产业化生产。

[0037] (3)本发明对病毒液的纯化方案为：通过DOE进行试验设计，通过大量实验数据得出寨卡病毒最优培养基，病毒液超滤浓缩、密度梯度离心、离子交换等纯化方法步骤简单同时能够利用密度梯度的优势简便快捷的纯化出具有免疫原性的完整病毒，有效地将含有E蛋白、M蛋白和C蛋白的完整寨卡病毒颗粒，此方法较层析方法即简便又经济，不仅提高了单位剂量寨卡病毒液的免疫原性而且降低了以此病毒液制备得到的疫苗使用后的副反应，同时降低了生产成本，且蔗糖密度梯度离心简便的操作过程和低廉的生产成本具有产业化可行性，同时相比层析方法能较好地去除杂质蛋白，保证了疫苗的安全性。

附图说明

[0038] 图1为实施例3病毒液密度梯度离心后的SDS PAGE电泳结果图。1-9泳道分别代表密度梯度离心收集的第10、11、12、13、14、15、16、17、18管离心液。其中第10-16管主要为杂质蛋白，包括牛血清残留蛋白，宿主细胞蛋白，而第17-18管为目标病毒，包含有寨卡病毒的3个结果蛋白E、M、C蛋白。

[0039] 图2为寨卡病毒原液HPLC检测结果。

具体实施方式

[0040] 以下实施例进一步说明本发明的内容，但不应理解为对本发明的限制。在不背离本发明精神和实质的情况下，对本发明方法、步骤或条件所作的修改或替换，均属于本发明的范围。

[0041] 若未特别指明，实施例中所用的技术手段为本领域技术人员所熟知的常规手段；实施例中所用的试剂为市售商品。

[0042] 本发明寨卡病毒来自浙江省疾病预防控制中心。

[0043] 实施例1 细胞工作种子的制备与病毒液的制备

[0044] 1、高密度细胞工作种子制备

[0045] 使用199培养液稀释Vero主细胞种子稀释比例为60:1，接种到175cm^{2}细胞方瓶中培养，培养温度为36.0℃±1℃。待细胞铺满细胞瓶，按1:4～1:6继续传代4代。最后一代传至5个40层细胞工厂，进行胰酶消化，离心去上清。使用含10％DMSO的冻存液进行重悬，混合后细胞浓度为2.5×10^{7}个/ml。将细胞种子在程序降温仪进行梯度降温，保持每分钟下降1℃。温度降至-196℃后移至液氮中保存，工作细胞种子的有效期为10年。

[0046] 2、Vero细胞培养

[0047] (1)一级细胞培养 称取微载体，用0.01M PBS溶胀过夜，用适量的PBS漂洗2次，置于高压蒸汽灭菌柜121℃灭菌。将细胞种子从液氮中取出，在39±1℃的水浴中迅速摇动融化，计数后，加入生长液中，进行一级细胞培养，培养体积约为5L。调整各培养参数，温度为36.5℃、pH值为7.20、溶氧为50％、转速为30rpm。细胞培养至平台期时2.5×10^{6}cells/ml，进行消化传代。

[0048] (2)二级细胞培养 将步骤(1)培养完成的细胞经消化制备成细胞悬液，接种至20L细胞反应器，调整各培养参数，温度为36.5℃、pH值为7.20、溶氧为50％、转速为30rpm，开始培养。细胞培养至平台期时5×10^{6}cells/ml，准备进行消化。

[0049] (3)三级细胞培养 步骤(2)培养完成后的细胞制备成细胞悬液，接种至工作体积120L细胞反应器中，设置温度为36.5℃、pH值为7.20、转速为30rpm、溶氧为50％。细胞密度培养至1.5×10^{6}cells/ml准备接种病毒。

[0050] 3、病毒液培养 按MOI 0.05寨卡病毒工作种子接种，将培养参数调整为转速：30rpm，温度：36.5℃，pH值：7.30，溶氧：50％。培养3天，收获细胞上清，即为寨卡病毒收获液。

[0051] 实施例2 培养基的筛选

[0052] 选择市面销售的MEM，M199，DMEM，DMEM/F12培养基，采用Minitab17的DOE软件进行试验设计，按照设计出的培养基配方进行病毒培养，比较病毒滴度、病毒免疫原性结果，选择最优培养基。试验设计的培养基配方如表1所示，其中“1”代表添加该培养基，“-1”代表不添加该培养基。配方中有几个“1”就进行几等分，每一种培养基分别为一等分。病毒滴度和免疫原性结果如表2，表3所示，其中M199与DMEM/F12按照1:1等体积混合的培养基生产的病毒免疫原性和滴度显著高于其它培养基。

[0053] 表1DOE试验培养基配方

[0054]

配方	MEM	M199	DMEM	DMEM/F12
①	-1	1	1	-1
②	1	1	1	1
③	1	-1	1	-1
④	1	1	-1	-1
⑤	-1	-1	1	1
⑥	1	-1	-1	1

[0055]

⑦	-1	1	-1	1

[0056] 表2病毒滴度结果

[0057]

配方	滴度结果($CCID_{50}$/ml)
①	7.0
②	6.5
③	6.9
④	7.3
⑤	7.1
⑥	6.8
⑦	7.9

[0058] 表3免疫原性结果

[0059]

配方	GMT(1:)
①	512
②	384
③	512
④	768
⑤	768
⑥	512
⑦	1024

[0060] 根据免疫原性和滴度结果选择⑦号培养基，作为寨卡病毒培养基。

[0061] 实施例3 寨卡病毒灭活和纯化

[0062] 1、寨卡病毒灭活 寨卡病毒收获液中添加终浓度为200μg/ml甲醛，置37℃灭活7天。即完成寨卡病毒的灭活。

[0063] 2、寨卡病毒纯化

[0064] (1)澄清 将病毒液经连续3级孔径为3-0.8μm、0.8-0.65μm、0.65-0.22μm的滤芯过滤或离心(包括连续流离心)转速为2000-5000rpm,离心时间为0.5-4个小时,得到病毒澄清液;

[0065] (2)浓缩 病毒澄清液经300KD截留分子量的Pall切向流膜过滤系统进行浓缩,浓缩倍数为100倍。

[0066] (3)密度梯度离心 蔗糖溶液的低、高浓度分别为25%,55%。密度梯度离心缓冲液为0.05mol/L PBS缓冲液,其pH值为:6.5。使用日立CP70MX/ME超速离心机,依次从边孔以100rpm泵入100ml超离心缓冲液,约700ml的实施例1超滤浓缩获得的寨卡病毒超滤液,低浓度蔗糖溶液500ml,最后以100rpm泵入高浓度蔗糖溶液直至中孔流出液体,在4℃经30000rpm超滤离心10h。离心完成后,第一管收集500ml,之后用50ml离心管收集,50ml/管,可以收集25-27管。检测每管的抗原含量和蛋白质含量,通过蛋白电泳结果合并含有E、M、C蛋白的离心液。各管的SDS PAGE电泳结果见图1,证明密度梯度离心可以将含有E、M、C蛋白的寨卡病毒有效分离。

[0067] (4)脱糖 步骤(3)中的密度梯度离心液经100KD截留分子量的Pall切向流膜过滤系统进行脱糖。

[0068] (5)离子交换 离子交换层析介质为DEAE FF,平衡缓冲液为0.02M PBS溶液,洗脱缓冲液为0.02M PBS,上样流速和清洗流速分别为15ml/min、80ml/min。收集流穿峰时以抗原峰向下出现拐点为界限,前后分为两段收集,即为层析液。

[0069] (6)纯化浓缩 步骤(5)中的层析液经100KD截留分子量的Pall切向流膜过滤系统进行浓缩,浓缩100倍后经0.22um过滤即为原液。原液通过HPLC检测纯度如图2所示,纯度达到98.6%。

[0070] 实施例4 寨卡病毒原液制备成疫苗的各项指标评价

[0071] 1、杂质含量此步骤产品即为寨卡病毒原液

[0072] 将实施例2获得的寨卡病毒原液根据抗原含量稀释至600U/ml,最终半成品的抗原含量为800U/ml,且总蛋白含量低于20μg/ml。随后加入;铝佐剂备成寨卡病毒疫苗成品。疫苗成品的杂质含量结果见表4。

[0073] 表4 6批寨卡病毒疫苗成品主要杂质残留量结果*

[0074]

寨卡病毒疫苗成品							
批次	1	2	3	4	5	6	中国药典
牛血清白蛋白残留（ng/剂）	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	≤50
Vero细胞蛋白质残留（ng/剂）	2	4	4	2	3	5	<1000
蛋白质含量（μg/剂）	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	-
DNA残留量（pg/剂）	<5	<5	<5	<5	<5	<5	≤100

[0075] *计算P值时如果数值为小于(<)值，按照该值进行计算。如果数值为范围值，按照范围上下限的平均值进行计算。

[0076] 2、免疫原性

[0077] 将疫苗成品复融后进行3倍系列稀释，共4个稀释度；每个稀释度样品分别进行单次两后肢肌肉免疫大鼠，0.5ml/只，10只(雌雄各半)每稀释度，免疫后21天采血，分离血清，分别检测血清中抗寨卡病毒的中和抗体滴度，计算血清阳转率。血清阳转率和中和抗体几何平均值GMT水平见表5。

[0078] 表5寨卡疫苗成品免疫原性结果

[0079]

批次	1	2	3	4	5	6
原倍阳转率	100%	100%	100%	100%	100%	100%
GMT(1:)	768	2048	1024	1536	1024	768

[0080] 3、稳定性

[0081] 将疫苗成品储存在37℃环境下，按时间点取样检测抗原含量、外观、pH值、渗透压摩尔浓度、细菌内毒素、BSA含量、HCP含量、DNA含量等。记录数据至8周，结果表6-11。

[0082] 表6 37℃成品-抗原含量检测结果(标示量的百分比)

批次	放置时间（周）抗原含量（%）*								
	0	1	2	3	4	5	6	7	8
1	100	97	101	95	95	90	86	84	60
2	100	101	102	98	97	88	82	74	55
3	100	106	104	99	91	85	79	72	67

[0083] *标示量的百分比值是较0月值的百分比值。

[0084] 表7 37℃DNA含量检测结果

[0085]

批次	放置时间（周）DNA 含量（pg/剂）					
	0	2	4	6	8	药典标准
1	<5	<5	<5	<5	<5	≤100
2	<5	<5	<5	<5	<5	≤100
3	<5	<5	<5	<5	<5	≤100
4	<5	<5	<5	<5	<5	≤100
5	<5	<5	<5	<5	<5	≤100
6	<5	<5	<5	<5	<5	≤100

[0086] 表8 37℃BSA含量检测结果

[0087]

批次	放置时间（周）BSA 含量（ng/剂）					
	0	2	4	6	8	药典标准
1	<2.5	<2.5	<2.5	<2.5	<2.5	≤50
2	<2.5	<2.5	<2.5	<2.5	<2.5	≤50
3	<2.5	<2.5	<2.5	<2.5	<2.5	≤50
4	<2.5	<2.5	<2.5	<2.5	<2.5	≤50
5	<2.5	<2.5	<2.5	<2.5	<2.5	≤50
6	<2.5	<2.5	<2.5	<2.5	<2.5	≤50

[0088] 表9 37℃HCP含量检测结果

[0089]

批次	放置时间（周）HCP 含量（ng/剂）					
	0	2	4	6	8	药典标准
1	2	3	3	4	2	≤1000
2	4	4	5	6	4	≤1000
3	4	4	4	4	4	≤1000
4	2	4	4	4	5	≤1000
5	3	4	4	5	5	≤1000
6	5	4	3	5	4	≤1000

[0090] 表10 37℃渗透压摩尔浓度检测结果

[0091]

批次	放置时间（周）渗透压摩尔浓度（mOsmol/kg）					
	0	2	4	6	8	药典标准
1	278	277	278	280	276	200-400
2	292	290	294	292	290	200-400
3	288	288	285	292	296	200-400
4	275	278	268	275	277	200-400
5	271	285	288	287	280	200-400

[0092]

6	286	288	289	286	288	200-400

[0093] 表11 37℃细菌内毒素检测结果

[0094]

批次	放置时间（周）细菌内毒素检查（EU/剂）					
	0	2	4	6	8	药典标准
1	≤5	≤5	≤5	≤5	≤5	≤5
2	≤5	≤5	≤5	≤5	≤5	≤5
3	≤5	≤5	≤5	≤5	≤5	≤5
4	≤5	≤5	≤5	≤5	≤5	≤5
5	≤5	≤5	≤5	≤5	≤5	≤5
6	≤5	≤5	≤5	≤5	≤5	≤5

[0095] 虽然，上文中已经用一般性说明及具体实施方案对本发明作了详尽的描述，但在本发明基础上，可以对之作一些修改或改进，这对本领域技术人员而言是显而易见的。因此，在不偏离本发明精神的基础上所做的这些修改或改进，均属于本发明要求保护的范围。

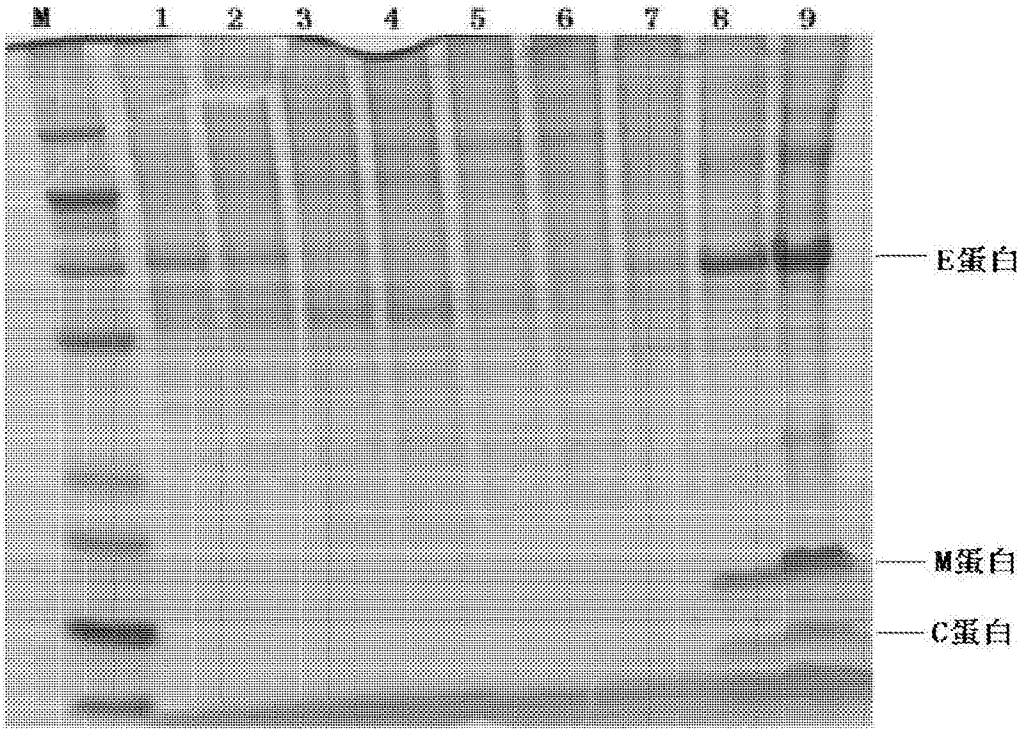
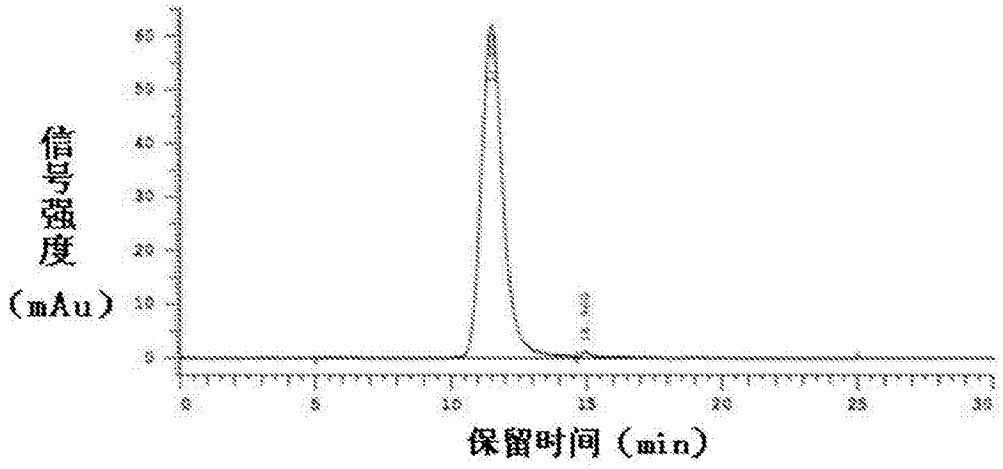


图1



No.	RT	面积	浓度1	BC	8-系数	10系数
1	11.480	1727814	98.584	BC		
2	14.960	24608	1.416	BC		
		1752422	100.000			

图2

Electronic Acknowledgement Receipt

EFS ID:	38117941
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
Filer:	Chelsea E. Witte-Garcia/Melissa Mann
Filer Authorized By:	Chelsea E. Witte-Garcia
Attorney Docket Number:	I0422.70125US00
Receipt Date:	23-DEC-2019
Filing Date:	15-JUN-2018
Time Stamp:	11:19:50
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	I042270125US00-TRN-CEW.pdf	26138 6d1ee4d07e1fe7c4327f5e61bdb76d13aa5efaab	no	1
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2	Transmittal Letter	I042270125US00-IDSBODY-CEW.pdf	32656 c97da8819786f3a2f378b5a7160dba3866fa09db	no	3
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3	Information Disclosure Statement (IDS) Form (SB08)	I042270125US00-IDS1449-CEW.pdf	52006 fa39d8e5d7695dd39811efe16eebd7c2dab73ccf	no	4
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4	Other Reference-Patent/App/Search documents	I042270125US00-ISR-mailed_4-10-17.pdf	166745 c1083c946bb4bde6dcb162f9bf9c6093aa384cec	no	5
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5	Other Reference-Patent/App/Search documents	I042270125US00-IPRP-mailed_6-26-18.pdf	363671 b9c7c0f89417fbf5ad4127682235ebcf80412e7f	no	15
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6	Foreign Reference	WO199911762A1.pdf	1528477 09d2b7e6ab218777d7f634fd56e049788e4a3ae9	no	34
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7	Foreign Reference	WO200192552A2.pdf	2570817 676d3429fa9e090934c6ccd8f854a61e37e0f321	no	46
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8	Foreign Reference	WO2013083726A1.pdf	13351701 8128cb9d867db6ef23cfd1b106acf79fdd6206ad	no	196
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9	Foreign Reference	WO2016145149A1.pdf	1737641 da6d065f4d6ca27f2f5fc5c2317ee5ab6557a3e4	no	32
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11	Foreign Reference	CN105749268_.pdf	889511 faddb93c0df72c373b8bdc9d993ca19158e65817	no	13
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12	Non Patent Literature	No_Author_listed_CDC_vaccines_7-2-2019_QC.pdf	71751 5a99985f2fd71cc07ccacd58efbdc16cc267650b	no	2
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13	Non Patent Literature	No_author_listed_CDCP_Japanese_encephalitis_vaccine_2016.pdf	108576 a98bfd93331c320fad78174ddcd3789f56dcd93b	no	3
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16	Non Patent Literature	No_Author_Listed_Wikimedia_Foundation_Inc_2015-qc.pdf	160651 892aca62df43944f70c955bc9ce64f8bdc75a615	no	2
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17	Non Patent Literature	No_Author_Listed_zika_virus_strain_h-pf-2013_2016.pdf	213484 251eff4d16667d92fc73e0bb7803ed1c64acaf5d	no	2
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18	Non Patent Literature	No_Author_listed_Pan-American_2015_.pdf	138206 bf3ed37bd641c26cf9f783797106b9e7d973b5a2	no	1
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19	Non Patent Literature	No_Author_listed_Valneva_press_release_7-7-2016_.pdf	233563 edd0d85be52622186e90749b20197473e577da10	no	3
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21	Non Patent Literature	Abbink_et_al_sci_trans_med_2017_v9_peaao4163.pdf	1416872 c62f183bd80a268bed987725490e4c26568e1dd0	no	7
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22	Non Patent Literature	Altschul_et_al_j_mol_biol_1990_v215_p403.pdf	713042 3a13153f033e188c8ccd86834b658e746efb7183	no	8
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23	Non Patent Literature	Altschul_et_al_nuc_acid_res_1997_v25_p3389.pdf	214624 03741de61b34eeb0f22e58e036aab5fbc1375029	no	14
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24	Non Patent Literature	Baronti_et_al_gen_ann_2014_v2_pe00500.pdf	192674 75cdc2252bbba6f3f8bf781142678d2329600f5b	no	2
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26	Non Patent Literature	Cox_et_al_anti_chem_chemo_2015_v24_p118.pdf	528773 a70a59c481d1bec1a6c2aee8943918ea536d8f35	no	9
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27	Non Patent Literature	Dowall_et_al_2016_plos.pdf	1202221 b0d05600668e7b114523ff30371f90dbab3a6983	no	13
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28	Non Patent Literature	Gardner_et_al_plos_neg_trop_2014_v8_pe2719.pdf	1705821 cab07e9f4235ae8dd421d1b62cbf5788bb47a8dc	no	16
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31	Non Patent Literature	Hallengard_et_al_2014_J_Virol_v88_p2858.pdf	1327623 6e78d70d037f3a4da1acd5822d59daf58e19bd6e	no	9
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33	Non Patent Literature	Hutornojs_et_al_env_exp_biol_2012_v10_p117.pdf	967689 23897c75aa65b0d3e87799ff3a34aefa4c3012df	no	7
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34	Non Patent Literature	Katoh_et_al_brief_bioinfo_2008_v9_p286.pdf	327573 66842300ddc444ab5305675ce4deef0391791666	no	13
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35	Non Patent Literature	Bender_et_al_valneva_world_2018_.pdf	2003790 1028fbea7f741a535b48616e019fdb402a9d37a3	no	26
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38	Non Patent Literature	Kofler_et_al_J_virol_2002_v76_p3534.pdf	1755004 e5f9d83e8ad234365d4f6dabfc008a66c17c0faa	no	10
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39	Non Patent Literature	Larkin_et_al_bioinfo_2007_v23_p2947.pdf	200047 442577c1da08b95a46c167c63ae7f444a9e9c96a	no	2
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43	Non Patent Literature	Needleman_et_al_j_mol_biol_1970_v48_p443.pdf	839217 27473d2aa4f5463c2dedb4083a054987de0b8906	no	11
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44	Non Patent Literature	Pearson_et_al_PNAS_1988_v85_p2444.pdf	1038136 d3efc6136a67471be162cac86e8de229e3775d95	no	5
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47	Non Patent Literature	Plevka_et_al_embo_rep_2011_v12_p602.pdf	1247599 1c3d227932dc8bb79dbd1b4f83c79671f04984d4	no	5
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48	Non Patent Literature	Putnak_et_al_J_Infect_Dis_1996_v174_p1176.pdf	2105356 8b6978d2df41c47819b2dfb61b6d2852b887e21a	no	9
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50	Non Patent Literature	Rocha_et_al_bull_world_health_2016.pdf	393062 5183254c57e57e849b78821f1b79bb6324fbf97d	no	9
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51	Non Patent Literature	Schlegl_et_al_vaccine_2015_v33_p5989.pdf	1378354 550cf19f22c65f57cf10b4e3abfbf29c5b944149	no	8
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52	Non Patent Literature	Smith_et_al_adv_appl_math_1981_v2_p482.pdf	351658 7df2cddeef9b95623f841d0d7687c5bf10f8f2fe	no	8
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Information:					
56	Non Patent Literature	Weaver_plos_2014_v8_p2921.pdf	556770 5af18ab9817865ec5ce157c5d6c26eb59c62b6f9	no	4
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57	Non Patent Literature	Waterhouse_et_al_bioinfo_2009_v25_p1189_.pdf	119808 c74bad27456783ffe82d6f522ce476868be23d98	no	3
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	1648
Examiner Name	B. A. Chestnut
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

PTO Form-1449
Copies of cited references

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Chelsea E. Witte-Garcia/		
Printed name	Chelsea E. Witte-Garcia, Ph.D.		
Date	December 23, 2019	Reg. No.	75,713

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Signature for Melissa Mann: /Melissa Mann/

DOCKET NO.: I0422.70125US00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	Barry A. Chestnut
Art Unit:	1648

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Signature Melissa Mann: /Melissa Mann/

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed before the mailing of a first Office Action on the merits in the above-identified case.

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the following co-pending applications that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
*16/060,054	06-07-2018	Barbero-Calzado et al.	I0422.70126US00
*15/781,825	06-06-2018	Barbero-Calzado et al.	I0422.70127US00

*A copy of this reference is not provided as the Office has waived the requirement under 37 C.F.R. § 1.98(a)(2)(iii) for submitting a copy of a cited U.S. patent application if it is scanned to the Image File Wrapper system and is available on Private PAIR .

The undersigned would like to bring to the Examiner's attention the enclosed search report or other communication from a corresponding or related International or Foreign National Application:

Serial No.	Date of Mailing	Type(s) of Communication
PCT/EP2016/082664	**04-10-2017**	**International Search Report and Written Opinion**
PCT/EP2016/082664	**06-26-2018**	**International Preliminary Report on Patentability**

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. I0422.70125US00 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Chelsea E. Witte-Garcia/
Chelsea E. Witte-Garcia, Ph.D.
Reg. No. 75,713
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: I0422.70125US00
Date: December 23, 2019

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00

CONFIRMATION NO. 4503

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

PUBLICATION NOTICE

OC000000114012791

Title:ZIKA VIRUS VACCINE

Publication No.US-2020-0017555-A9
Publication Date:01/16/2020

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 01/16/2020

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
CHESTNUT, BARRY A	

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
01/16/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Applicant-Initiated Interview Summary	**Application No.** 16/063,007	**Applicant(s)** BARBERO CALZADO et al.	
	Examiner Barry Chestnut	**Art Unit** 1648	**AIA (FITF) Status** Yes

All participants (applicant, applicants representative, PTO personnel):

(1) Barry Chestnut (PTO). (3) _____.

(2) Chelsea Witte-Garcia (Applicant's Representative). (4) _____.

Date of Interview: 13 January 2020.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☐ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 3.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The interview with Dr. Witte-Garcia in regards to application no. 16/063007 pertained to the Applicant's species election in claim 3. Particularly, the Applicant elects SEQ ID NO: 13 and not SEQ ID NO: 3. The Examiner will examine the claims with respect to SEQ ID NO: 13 in the first action on merit..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/BARRY A CHESTNUT/ Primary Examiner, Art Unit 1648	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
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www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

23628 7590 01/31/2020
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER
CHESTNUT, BARRY A

ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 01/31/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

TITLE OF INVENTION: ZIKA VIRUS VACCINE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	04/30/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

23628 7590 01/31/2020
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

TITLE OF INVENTION: ZIKA VIRUS VACCINE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	04/30/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHESTNUT, BARRY A	1648	424-218100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

PTOL-85 Part B (08-18) Approved for use through 01/31/2020 OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 01/31/2020

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
CHESTNUT, BARRY A	
ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 01/31/2020

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 16/063,007	Applicant(s) BARBERO CALZADO et al.	
	Examiner Barry Chestnut	Art Unit 1648	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to 03/20/2019.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1-20 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☑ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☑ All b) ☐ Some *c) ☐ None of the:

   1. ☑ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☑ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 12/23/2019.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/BARRY A CHESTNUT/
Primary Examiner, Art Unit 1648

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

SPECIFICATION

The disclosure is objected to because of the following informalities:

The disclosure is objected to because it contains an embedded hyperlink and/or other form of browser-executable code (see page 1 and page 86). Applicant is required to delete the embedded hyperlink and/or other form of browser-executable code; references to websites should be limited to the top-level domain name without any prefix such as http:// or other browser-executable code. See MPEP § 608.01.

Appropriate correction is required.

Examiner's Statement of Reasons for Allowance

The Examiner's statement of reasons for allowance is the following: the claims are directed to a Zika virus vaccine comprising an inactivated Zika virus wherein the Zika virus vaccine contains protamine sulphate (PS) or fragments or break-down products of PS at amounts below 1 ug/mL and wherein the vaccine is able to confer seroprotection on at least 70% of subjects that are administered the Zika virus vaccine that is free of the prior art of record. The closest prior art, citing Cox et al. (Antiviral chemistry & Chemotherapy, 24(3-4), 2015:118-126), discloses that Zika virus is in the center of an emerging crisis that requires the development of a vaccine. Cox discloses that lead candidates of treatment can likely be established using NS3 and NS5 inhibitors from other flaviviruses, and the structures presented can provide opportunities for Zika virus intervention strategies (see Abstract). Cox does not relate to an inactivated Zika virus vaccine comprising a protamine sulphate or fragment at amounts below 1 ug/mL.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Barry Chestnut whose telephone number is 571-270-3546. The examiner can normally be reached on M-F 8 am-4 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Janet Andres can be reached on 571-272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/BARRY A CHESTNUT/
Primary Examiner, Art Unit 1648

Notice of References Cited	Application/Control No. 16/063,007	Applicant(s)/Patent Under Reexamination BARBERO CALZADO et al.	
	Examiner Barry Chestnut	Art Unit 1648	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A					
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Cox et al., "Predicting Zika virus structural biology: Challenges and opportunities for intervention", Antiviral chemistry & chemotherapy, 24(3-4), 2015:118-126.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/063,007	BARBERO CALZADO et al.
	Examiner	Art Unit
	Barry Chestnut	1648

CPC - Searched*		
Symbol	Date	Examiner
A61K2039/53,A61K2039/55555,A61K39/12,A61K39/39,A61K48/00	01/15/2020	BC / AU 1648

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor search- Barbero, J., Nebenfuhr, M., Schlgel, R., Weber, M., Heindl, J. (PALM/EAST all inventors) STN	01/15/2020	BC / AU 1648
NPL search- attached- Medline, Biosis, Caplus, Scisearch, Embase	01/15/2020	BC / AU 1648

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
US PGPUB		01/15/2020	BC / AU 1648
UPAD		01/15/2020	BC / AU 1648

/BARRY A CHESTNUT/ Primary Examiner, Art Unit 1648	01/15/2020

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/063,007	BARBERO CALZADO et al.
	Examiner	**Art Unit**
	Barry Chestnut	1648

CPC						
Symbol					**Type**	**Version**
A61K	/	39	/	12	F	2013-01-01
A61P	/	31	/	14	I	2018-01-01
C07K	/	14	/	1825	I	2013-01-01
C12N	/	7	/	02	I	2013-01-01
C12N	/	7	/	06	I	2013-01-01
C12N	/	7	/	00	I	2013-01-01
A61K	/	39	/	39	I	2013-01-01
C07K	/	14	/	18	I	2013-01-01
A61K	/	2039	/	5252	A	2013-01-01
C12N	/	2770	/	24134	A	2013-01-01
C12N	/	2770	/	24151	A	2013-01-01
C12N	/	2770	/	24163	A	2013-01-01
Y02A	/	50	/	386	A	2018-01-01
Y02A	/	50	/	388	A	2018-01-01
Y02A	/	50	/	383	A	2018-01-01
Y02A	/	50	/	392	A	2018-01-01
Y02A	/	50	/	39	A	2018-01-01
A61K	/	2039	/	5258	A	2013-01-01
A61K	/	2039	/	5254	A	2013-01-01
A61K	/	2039	/	55505	A	2013-01-01

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 20	
/BARRY A CHESTNUT/ Primary Examiner, Art Unit 1648 (Primary Examiner)	15 January 2020 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/063,007	BARBERO CALZADO et al.
	Examiner	**Art Unit**
	Barry Chestnut	1648

INTERNATIONAL CLASSIFICATION				
CLAIMED				
A61K	/	39	/	00
A61K	/	39	/	12
A61K	/	39	/	39
A61K	/	48	/	00
A61K	/	9	/	00

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	20	
/BARRY A CHESTNUT/ Primary Examiner, Art Unit 1648	15 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/063,007	BARBERO CALZADO et al.
	Examiner	**Art Unit**
	Barry Chestnut	1648

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	20	
/BARRY A CHESTNUT/ Primary Examiner, Art Unit 1648	15 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office

Part of Paper No.: 20200115

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	5	((("BARBERO") near3 ("CALZADO") near3 ("Jana"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:03
L2	0	((("NEBENFÃœHR") near3 ("Mario"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:03
L3	0	nebenfuhr near2 mario.inv.	USPAT	OR	OFF	2020/01/15 16:07
L4	1	((("SCHLGEL") near3 ("Robert"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:08
L5	814	((("WEBER") near3 ("Michael"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:08
L6	0	((("HEINDL-WRUSS") near3 ("JÃ¼rgen"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:08
L7	422	l1 or l3 or l4 or l5 or l6	USPAT	OR	OFF	2020/01/15 16:09
L8	0	"zika virus" and "protamine sulphate"	USPAT	OR	OFF	2020/01/15 16:10
L9	237	"zika virus"	USPAT	OR	OFF	2020/01/15 16:10
L10	125	l9 and vaccine	USPAT	OR	OFF	2020/01/15 16:11
L11	5	l10 and seroprotection	USPAT	OR	OFF	2020/01/15 16:11

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	barbero near2 calzado.inv.	USPAT	OR	OFF	2020/01/15 16:25
L2	0	barbero near3 jana.inv.	USPAT	OR	OFF	2020/01/15 16:25
L3	0	nebenfuhr near2 mario.inv.	USPAT	OR	OFF	2020/01/15 16:26
L4	0	schlgel near2 robert.inv.	USPAT	OR	OFF	2020/01/15 16:26
L5	422	weber near2 michael.inv.	USPAT	OR	OFF	2020/01/15 16:26
L6	0	heindl near3 jurgen.inv.	USPAT	OR	OFF	2020/01/15 16:26
L7	422	l1 or l2 or l3 or l4 or l5 or l6	USPAT	OR	OFF	2020/01/15 16:27
L8	0	"zika virus" and protamine.clm.	USPAT	OR	OFF	2020/01/15 16:27

Bibliographic Data

Application No: 16/063,007

Foreign Priority claimed: (•) Yes ( ) No

35 USC 119 (a-d) conditions met: [✓] Yes [ ] No [ ] Met After Allowance

Verified and Acknowledged: /BARRY A CHESTNUT/ Examiner's Signature [ ] Initials

Title: ZIKA VIRUS VACCINE

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
06/15/2018	424	1648	I0422.70125US00
RULE			

APPLICANTS

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Jürgen HEINDL-WRUSS Vienna, AUSTRIA

CONTINUING DATA

This application is a 371 of PCT/EP2016/082664 12/23/2016

FOREIGN APPLICATIONS

EPO 15202585.4 12/23/2015

EPO 16161068.8 03/18/2016

EPO 16176049.1 06/23/2016

EPO 16176025.1 06/23/2016

EPO 16182845.4 08/04/2016

IF REQUIRED, FOREIGN LICENSE GRANTED**

09/19/2018

STATE OR COUNTRY

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BOSTON, MA 02210-2206

UNITED STATES

FILING FEE RECEIVED

$1,980

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	1	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document Number	U.S. Patent Document Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		6,309,650	B1	Kim et al.	10-30-2001
		8,765,148	B2	Wizel et al.	07-01-2014
		10,086,061	B2	Thomas et al.	10-02-2018
		2013-0280295	A1	Schlegl et al.	10-24-2013
		2018-0362936	A1	Barbero Calzado et al.	12-20-2018
		2019-0008945	A1	Barbero Calzado et al.	01-10-2019

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document Office/ Country	Foreign Patent Document Number	Foreign Patent Document Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		CN	105749268	A	Sinovac Research and Development Co Ltd.	07-13-2016	Y-Abstract
		WO	1999/011762	A1	Cheil Jedang Corporation, et al.	03-11-1999	
		WO	2001/092552	A2	Chiron Corporation	12-06-2001	
		WO	2013/083726	A1	Intercell AG	06-13-2013	
		WO	2016/145149	A1	The United States of America, as represented by the Secretary of the Army, on Behalf of the Walter Reed Army Institute of Research	09-15-2016	
		WO	2017/009873	A1	Bharat Biotech International Limited	01-19-2017	

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		[NO AUTHOR LISTED] Centers for Disease Control and Prevention Ingredients of vaccines fact sheet. Retrieved from https://www.cdc.gov/vaccines/vac-gen/additives.htm.	
		[NO AUTHOR LISTED] Centers for Disease Control and Prevention. 2016. Japanese Encephalitis Vaccine. Retrieved from https://www.cdc.gov/japaneseencephalitis/vaccine/ on 16-Jun-2016.	
		[NO AUTHOR LISTED] Genbank Accession No. ABI54475. polyprotein [Zika virus]. 4 pages. December 24, 2009.	
		[NO AUTHOR LISTED] Media centre. Zika virus. World Health Organization, 2016. Zika Virus Fact Sheet. Downloaded March 11, 2016 from http://www.who.int/en/news-room/fact-sheets/detail/zika-virus.	

EXAMINER:	DATE CONSIDERED:
/BARRY A CHESTNUT/	01/15/2020

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

6565846.1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.A.C/

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	2	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

		[NO AUTHOR LISTED] Pan-American Health Organization. 2015. Number of Reported Cases of Chikungunya Fever in the Americas, by Country or Territory 2013-2014, Cumulative Cases (Updated 23-Oct-2015).	
		[NO AUTHOR LISTED] Valneva Announces Successful Generation of a Highly-purified Zika Vaccine Candidate Using its FDA-EMA Approved Japanese Encephalitis Platform. Press release July 7, 2016.	
		[NO AUTHOR LISTED] Wikimedia Foundation, Inc., 2015. https://en.wikipedia.org/wiki/Protamine_sulfate; updated 30-Sept-2015; downloaded 26-Nov-2015.	
		[NO AUTHOR LISTED] World Health Organization, 2016. Zika Situation Report 05-Feb-2016.	
		[NO AUTHOR LISTED] Zika virus, strain H/PF/2013. November 28, 2013. European Virus Archive retrieved on December 22, 2016 from http://www.who.int/mediacentre/factsheets/zika/en.	
		ABBINK et al, Durability and correlates of vaccine protection against Zika virus in rhesus monkeys. Sci. Transl. Med. 2017;9:eaao4163.	
		ALTSCHUL et al., Basic Local Alignment Search Tool. J. Mol. Biol. 1990;215:403-410.	
		ALTSCHUL et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nuc. Acids Res. 1997;25:3389-3402.	
		BARONTI et al., Complete Coding Sequence of Zika Virus from a French Polynesia Outbreak in 2013. Genome Announc. 2014 May-Jun; 2(3):e00500-14. Abstract.	
		BENDER et al., Zika Virus Vaccine Candidate VLA1601: Cooperation VALNEVA & EMERGENT, Presentation at World Vaccine Congress April 4, 2018.	
		COHEN, INFECTIOUS DISEASE. The race for a Zika vaccine is on. Science. 2016 Feb 5;351(6273):543-4. doi: 10.1126/science.351.6273.543.	
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EXAMINER:	DATE CONSIDERED:
/BARRY A CHESTNUT/	01/15/2020

[#] EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.A.C/

FORM PTO-1449/A and B (modified PTO/SB/08) **INFORMATION DISCLOSURE STATEMENT BY APPLICANT**				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	3	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

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EXAMINER:	DATE CONSIDERED:
/BARRY A CHESTNUT/	01/15/2020

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FORM PTO-1449/A and B (modified PTO/SB/08) **INFORMATION DISCLOSURE STATEMENT BY APPLICANT**				APPLICATION NO.: 16/063,007	ATTY. DOCKET NO.: I0422.70125US00
				FILING DATE: June 15, 2018	CONFIRMATION NO.: 4503
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	4	of	4	GROUP ART UNIT: 1648	EXAMINER: Barry A. Chestnut

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/BARRY A CHESTNUT/	01/15/2020

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L2           5 L1 AND ZIKA

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L3            5 DUP REM L2 (0 DUPLICATES REMOVED)

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L3   ANSWER 1 OF 5  CAPLUS  COPYRIGHT 2020 ACS on STN
TI   Zika virus particles purification using a protamine salt.
ACCESSION NUMBER:        2017:1072994  CAPLUS Full-text
DOCUMENT NUMBER:         167:124812
TITLE:                   Zika virus particles purification using a protamine
                         salt.
INVENTOR(S):             Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl,
                         Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S):      Valneva Austria GmbH, Austria
SOURCE:                  PCT Int. Appl., 120pp.
                         CODEN: PIXXD2
DOCUMENT TYPE:           Patent
LANGUAGE:                English
FAMILY ACC. NUM. COUNT:  6
PATENT INFORMATION:
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EP 3393508	A1	20181031	EP 2016-826741		20161223
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			EP 2016-182845	A	20160804
			WO 2016-EP82667	W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072994

L3 ANSWER 2 OF 5 CAPLUS COPYRIGHT 2020 ACS on STN
TI Zika virus: purification and protective vaccination
ACCESSION NUMBER: 2017:1072972 CAPLUS Full-text
DOCUMENT NUMBER: 167:124811
TITLE: Zika virus: purification and protective vaccination
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 140pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.		DATE
WO 2017109227	A1	20170629	WO 2016-EP82666		20161223
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			EP 2016-176025	A	20160623
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			WO 2016-EP82666	W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072972

L3 ANSWER 3 OF 5 CAPLUS COPYRIGHT 2020 ACS on STN
TI Zika virus vaccine

ACCESSION NUMBER: 2017:1072945 CAPLUS Full-text
DOCUMENT NUMBER: 167:124810
TITLE: Zika virus vaccine
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 130pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

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EP 3393510	A1	20181031	EP 2016-828746	20161223
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US 20180371027	A1	20181227	US 2018-16063007	20180615
IN 201817022751	A	20181102	IN 2018-17022751	20180618

PRIORITY APPLN. INFO.:

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WO 2016-EP82664	W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072945

L3 ANSWER 4 OF 5 CAPLUS COPYRIGHT 2020 ACS on STN
TI Virus purification
ACCESSION NUMBER: 2017:1072906 CAPLUS Full-text
DOCUMENT NUMBER: 167:133267
TITLE: Virus purification
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva SE, Fr.
SOURCE: PCT Int. Appl., 161pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

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EP 3393506	A1	20181031	EP 2016-826739	20161223
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US 20180362937	A1	20181220	US 2018-15781959	20180606
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			EP 2016-161068 A	20160318
			EP 2016-176025 A	20160623
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			WO 2016-EP82663 W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072906
REFERENCE COUNT: 5 THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L3 ANSWER 5 OF 5 CAPLUS COPYRIGHT 2020 ACS on STN
TI Virus purification
ACCESSION NUMBER: 2017:1072907 CAPLUS Full-text
DOCUMENT NUMBER: 167:158066
TITLE: Virus purification
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva SE, Fr.
SOURCE: PCT Int. Appl., 184pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109223	A1	20170629	WO 2016-EP82662	20161223
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM				
EP 3393509	A1	20181031	EP 2016-828745	20161223
R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,				

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            RS, SE, SI, SK, SM, TR, BA, ME
     US 20180369359       A1   20181227    US 2018-16062245        20180614
PRIORITY APPLN. INFO.:                     EP 2015-202585       A  20151223
                                           EP 2016-161068       A  20160318
                                           EP 2016-176025       A  20160623
                                           EP 2016-176049       A  20160623
                                           EP 2016-182845       A  20160804
                                           WO 2016-EP82662      W  20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S):         CASFORMULTNS 2017:1072907
OS.CITING REF COUNT:     1     THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD
                               (1 CITINGS)
REFERENCE COUNT:         8     THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS
                               RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT


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E6           65     NEBENFUHRER L/AU
E7            6     NEBENFUHRER LASZLO/AU
E8            8     NEBENFUHRER Z/AU
E9            6     NEBENFUHRER ZSUZSA/AU
E10           2     NEBENFUHRER ZSUZSANNA/AU
E11           8     NEBENIUS OOSTHUIZEN D/AU
E12           9     NEBENIUS OOSTHUIZEN DANIELA/AU

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E1            1     SCHLFTER D/AU
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E10           2     SCHLIACK/AU
E11           5     SCHLIACK E/AU
E12         213     SCHLIACK H/AU

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L5            0 !SCHLGEL ROBERT!/AU

=> e weber michael/au
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E2            1     WEBER MICHAEEL PHILIPPE/AU
E3         2526 --> WEBER MICHAEL/AU
E4         1021     WEBER MICHAEL A/AU
E5           53     WEBER MICHAEL A DR/AU
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E7            1     WEBER MICHAEL A M D/AU
E8           12     WEBER MICHAEL A MD/AU
E9            3     WEBER MICHAEL A PROF/AU
E10           1     WEBER MICHAEL A PROF DR/AU
E11         155     WEBER MICHAEL D/AU
E12           1     WEBER MICHAEL DAVID/AU
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L6         2526 !WEBER MICHAEL!/AU

=> s l6 and zika
L7            5 L6 AND ZIKA

=> dup rem l7
PROCESSING COMPLETED FOR L7
L8             5 DUP REM L7 (0 DUPLICATES REMOVED)

=> disp l8 1-5 ti ibib

L8   ANSWER 1 OF 5  CAPLUS  COPYRIGHT 2020 ACS on STN
TI   Zika virus particles purification using a protamine salt.
ACCESSION NUMBER:         2017:1072994  CAPLUS Full-text
DOCUMENT NUMBER:          167:124812
TITLE:                    Zika virus particles purification using a protamine
                          salt.
INVENTOR(S):              Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl,
                          Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S):       Valneva Austria GmbH, Austria
SOURCE:                   PCT Int. Appl., 120pp.
                          CODEN: PIXXD2
DOCUMENT TYPE:            Patent
LANGUAGE:                 English
FAMILY ACC. NUM. COUNT:   6
PATENT INFORMATION:
     PATENT NO.           KIND  DATE        APPLICATION NO.          DATE
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     WO 2017109228         A1   20170629    WO 2016-EP82667          20161223
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             EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,
             IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS,
             LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
             NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD,
             SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA,
             UG, US, UZ, VC, VN, ZA, ZM, ZW
         RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
             HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS,
             SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM,
             ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW,
             SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM
     EP 3393508            A1   20181031    EP 2016-826741           20161223
         R:  AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
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             RS, SE, SI, SK, SM, TR, BA, ME
     US 20180362936        A1   20181220    US 2018-15781825         20180606
PRIORITY APPLN. INFO.:                     EP 2015-202585       A   20151223
                                           EP 2016-161068       A   20160318
                                           EP 2016-176025       A   20160623
                                           EP 2016-176049       A   20160623
                                           EP 2016-182845       A   20160804
                                           WO 2016-EP82667      W   20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S):          CASFORMULTNS 2017:1072994

L8   ANSWER 2 OF 5  CAPLUS  COPYRIGHT 2020 ACS on STN
TI   Zika virus: purification and protective vaccination
ACCESSION NUMBER:         2017:1072972  CAPLUS Full-text
DOCUMENT NUMBER:          167:124811
TITLE:                    Zika virus: purification and protective vaccination
INVENTOR(S):              Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl,
```

Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 140pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109227	A1	20170629	WO 2016-EP82666	20161223
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM				
EP 3393507	A1	20181031	EP 2016-826740	20161223
R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BA, ME				
US 20190008945	A1	20190110	US 2018-16060054	20180607

PRIORITY APPLN. INFO.:

EP 2015-202585	A	20151223
EP 2016-161068	A	20160318
EP 2016-176025	A	20160623
EP 2016-176049	A	20160623
EP 2016-182845	A	20160804
WO 2016-EP82666	W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072972

L8 ANSWER 3 OF 5 CAPLUS COPYRIGHT 2020 ACS on STN
TI Zika virus vaccine
ACCESSION NUMBER: 2017:1072945 CAPLUS Full-text
DOCUMENT NUMBER: 167:124810
TITLE: Zika virus vaccine
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 130pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109225	A1	20170629	WO 2016-EP82664	20161223
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,				

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SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM,
ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW,
SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM

CA 3009278	A1	20170629	CA 2016-3009278	20161223
AU 2016375338	A1	20180705	AU 2016-375338	20161223
KR 2018097558	A	20180831	KR 2018-7017319	20161223
CN 108697785	A	20181023	CN 2016-80074939	20161223
EP 3393510	A1	20181031	EP 2016-828746	20161223

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HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO,
RS, SE, SI, SK, SM, TR, BA, ME

BR 112018012873	A2	20181204	BR 2018-112018012873	20161223
JP 2019504030	T	20190214	JP 2018-532747	20161223
MX 2018007627	A	20190214	MX 2018-7627	20161223
US 20180371027	A1	20181227	US 2018-16063007	20180615
IN 201817022751	A	20181102	IN 2018-17022751	20180618

PRIORITY APPLN. INFO.:

EP 2015-202585	A	20151223
EP 2016-161068	A	20160318
EP 2016-176025	A	20160623
EP 2016-176049	A	20160623
EP 2016-182845	A	20160804
WO 2016-EP82664	W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072945

L8 ANSWER 4 OF 5 CAPLUS COPYRIGHT 2020 ACS on STN
TI Virus purification
ACCESSION NUMBER: 2017:1072906 CAPLUS Full-text
DOCUMENT NUMBER: 167:133267
TITLE: Virus purification
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva SE, Fr.
SOURCE: PCT Int. Appl., 161pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109224	A1	20170629	WO 2016-EP82663	20161223

W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
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EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,
IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS,
LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, ZA, ZM, ZW

RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS,
SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM,
ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW,
SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM

EP 3393506	A1	20181031	EP 2016-826739	20161223

R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
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RS, SE, SI, SK, SM, TR, BA, ME

US 20180362937	A1	20181220	US 2018-15781959	20180606

PRIORITY APPLN. INFO.:

EP 2015-202585	A	20151223
EP 2016-161068	A	20160318
EP 2016-176025	A	20160623

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                                        WO 2016-EP82663      W  20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S):         CASFORMULTNS 2017:1072906
REFERENCE COUNT:         5     THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS
                               RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L8   ANSWER 5 OF 5  CAPLUS  COPYRIGHT 2020 ACS on STN
TI   Virus purification
ACCESSION NUMBER:        2017:1072907  CAPLUS Full-text
DOCUMENT NUMBER:         167:158066
TITLE:                   Virus purification
INVENTOR(S):             Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl,
                         Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S):      Valneva SE, Fr.
SOURCE:                  PCT Int. Appl., 184pp.
                         CODEN: PIXXD2
DOCUMENT TYPE:           Patent
LANGUAGE:                English
FAMILY ACC. NUM. COUNT:  6
PATENT INFORMATION:
     PATENT NO.          KIND  DATE        APPLICATION NO.          DATE
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     WO 2017109223        A1   20170629    WO 2016-EP82662          20161223
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             EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,
             IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS,
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             NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD,
             SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA,
             UG, US, UZ, VC, VN, ZA, ZM, ZW
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             SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM
     EP 3393509           A1   20181031    EP 2016-828745           20161223
         R:  AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
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             RS, SE, SI, SK, SM, TR, BA, ME
     US 20180369359       A1   20181227    US 2018-16062245         20180614
PRIORITY APPLN. INFO.:                    EP 2015-202585       A  20151223
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                                          WO 2016-EP82662      W  20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S):         CASFORMULTNS 2017:1072907
OS.CITING REF COUNT:     1     THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD
                               (1 CITINGS)
REFERENCE COUNT:         8     THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS
                               RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT


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E6            1     HEINDLE STEPHANIE/AU
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E9           11     HEINDLER FRANZ M/AU
E10          54     HEINDLER M/AU
E11           6     HEINDLER MANFRED/AU
E12           4     HEINDLER NINA/AU

=> s e3
L9            0 !HEINDL WRUSS JURGEN!/AU

=> e zika
E1            2     ZIK8/BI
E2            1     ZIK9/BI
E3        33464 --> ZIKA/BI
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E5            2     ZIKAAFFECTED/BI
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=> s e3
L10       33464 ZIKA/BI

=> s l10 and virus
L11       30899 L10 AND VIRUS

=> s l11 and vaccine
L12        4915 L11 AND VACCINE

=> s l12 and protamine
L13           6 L12 AND PROTAMINE

=> dup rem l13
PROCESSING COMPLETED FOR L13
L14           6 DUP REM L13 (0 DUPLICATES REMOVED)

=> disp l14 1-6 ti ibib

L14  ANSWER 1 OF 6  EMBASE  COPYRIGHT (c) 2020 Elsevier B.V. All rights
     reserved on STN
TI   mRNA vaccines-a new era in vaccinology.
ACCESSION NUMBER:   20180230586  EMBASE   Full-text
DOCUMENT NUMBER:    621499599  PMID: 29326426
TITLE:              mRNA vaccines-a new era in vaccinology.
AUTHOR:             Pardi, Norbert; Hogan, Michael J.; Weissman, Drew
                    (correspondence)
CORPORATE SOURCE:   Department of Medicine, University of Pennsylvania,
                    Philadelphia, PA, 19104, United States. dreww@upenn.edu
AUTHOR:             Porter, Frederick W.
CORPORATE SOURCE:   Duke Human Vaccine Institute, Duke University, School of
                    Medicine, Durham, NC, 27710, United States.
SOURCE:             Nature Reviews Drug Discovery, (28 Mar 2018) Vol. 17, No.
                    4, pp. 261-279.
                    Refs: 222
                    ISSN: 1474-1776; E-ISSN: 1474-1784  CODEN: NRDDAG
DIGITAL OBJECT ID:  10.1038/nrd.2017.243
PUBLISHER:          Nature Publishing Group, Houndmills, Basingstoke,
                    Hampshire, RG21 6XS, United Kingdom.
COUNTRY:            United Kingdom
DOCUMENT TYPE:      Journal; General Review; (Review)
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FILE SEGMENT: Intellectual Indexing
016 Cancer
022 Human Genetics
029 Clinical and Experimental Biochemistry
037 Drug Literature Index
039 Pharmacy
004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
FILE SEGMENT:ClinicalTrials.gov
CLINICAL TRIAL NO.: NCT00087984; NCT00228189; NCT00243529; NCT00272649; NCT00381212; NCT00510133; NCT00514189; NCT00626483; NCT00639639; NCT00664482; NCT00672191; NCT00678119; NCT00831467; NCT00833781; NCT00834002; NCT00846456; NCT00890032; NCT00923312; NCT00929019; NCT00940004; NCT00961844; NCT00965224; NCT00978913; NCT01066390; NCT01069809; NCT01197625; NCT01216436; NCT01278914; NCT01278940; NCT01291420; NCT01302496; NCT01334047; NCT01446731; NCT01456065; NCT01456104; NCT01482949; NCT01530698; NCT01582672; NCT01676779; NCT01684241; NCT01686334; NCT01734304; NCT01817738; NCT01915524; NCT01983748; NCT01995708; NCT02035956; NCT02042248; NCT02140138; NCT02241135; NCT02285413; NCT02316457; NCT02366728; NCT02410733; NCT02413645; NCT02529072; NCT02649582; NCT02649829; NCT02709616; NCT02808364; NCT02808416; NCT02888756; NCT03014089; NCT03076385
LANGUAGE: English
SUMMARY LANGUAGE: English
ENTRY DATE: Entered Embase: 5 Apr 2018
Last Updated on Embase: 24 Jul 2019
Indexing Added: 12 Apr 2018
First Entered Embase or Embase Alert: 5 Apr 2018

L14 ANSWER 2 OF 6 CAPLUS COPYRIGHT 2020 ACS on STN
TI Zika virus particles purification using a protamine salt.
ACCESSION NUMBER: 2017:1072994 CAPLUS Full-text
DOCUMENT NUMBER: 167:124812
TITLE: Zika virus particles purification using a protamine salt.
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 120pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109228	A1	20170629	WO 2016-EP82667	20161223
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM				
EP 3393508	A1	20181031	EP 2016-826741	20161223

R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BA, ME

US 20180362936 A1 20181220 US 2018-15781825 20180606
PRIORITY APPLN. INFO.: EP 2015-202585 A 20151223
EP 2016-161068 A 20160318
EP 2016-176025 A 20160623
EP 2016-176049 A 20160623
EP 2016-182845 A 20160804
WO 2016-EP82667 W 20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072994

L14 ANSWER 3 OF 6 CAPLUS COPYRIGHT 2020 ACS on STN
TI Zika virus: purification and protective vaccination
ACCESSION NUMBER: 2017:1072972 CAPLUS Full-text
DOCUMENT NUMBER: 167:124811
TITLE: Zika virus: purification and protective vaccination
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 140pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109227	A1	20170629	WO 2016-EP82666	20161223

W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM

EP 3393507 A1 20181031 EP 2016-826740 20161223
R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BA, ME

US 20190008945 A1 20190110 US 2018-16060054 20180607
PRIORITY APPLN. INFO.: EP 2015-202585 A 20151223
EP 2016-161068 A 20160318
EP 2016-176025 A 20160623
EP 2016-176049 A 20160623
EP 2016-182845 A 20160804
WO 2016-EP82666 W 20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072972

L14 ANSWER 4 OF 6 CAPLUS COPYRIGHT 2020 ACS on STN
TI Zika virus vaccine
ACCESSION NUMBER: 2017:1072945 CAPLUS Full-text
DOCUMENT NUMBER: 167:124810
TITLE: Zika virus vaccine
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen

PATENT ASSIGNEE(S): Valneva Austria GmbH, Austria
SOURCE: PCT Int. Appl., 130pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109225	A1	20170629	WO 2016-EP82664	20161223
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW				
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM				
CA 3009278	A1	20170629	CA 2016-3009278	20161223
AU 2016375338	A1	20180705	AU 2016-375338	20161223
KR 2018097558	A	20180831	KR 2018-7017319	20161223
CN 108697785	A	20181023	CN 2016-80074939	20161223
EP 3393510	A1	20181031	EP 2016-828746	20161223
R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, BA, ME				
BR 112018012873	A2	20181204	BR 2018-112018012873	20161223
JP 2019504030	T	20190214	JP 2018-532747	20161223
MX 2018007627	A	20190214	MX 2018-7627	20161223
US 20180371027	A1	20181227	US 2018-16063007	20180615
IN 201817022751	A	20181102	IN 2018-17022751	20180618

PRIORITY APPLN. INFO.:

EP 2015-202585	A	20151223
EP 2016-161068	A	20160318
EP 2016-176025	A	20160623
EP 2016-176049	A	20160623
EP 2016-182845	A	20160804
WO 2016-EP82664	W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072945

L14 ANSWER 5 OF 6 CAPLUS COPYRIGHT 2020 ACS on STN
TI Virus purification
ACCESSION NUMBER: 2017:1072906 CAPLUS Full-text
DOCUMENT NUMBER: 167:133267
TITLE: Virus purification
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva SE, Fr.
SOURCE: PCT Int. Appl., 161pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109224	A1	20170629	WO 2016-EP82663	20161223
W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC,				

EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,
IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS,
LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, ZA, ZM, ZW
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS,
SE, SI, SK, SM, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM,
ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW,
SD, SL, ST, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, RU, TJ, TM

EP 3393506	A1	20181031	EP 2016-826739	20161223

R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
HU, IE, IS, IT, LI, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO,
RS, SE, SI, SK, SM, TR, BA, ME

US 20180362937	A1	20181220	US 2018-15781959	20180606
PRIORITY APPLN. INFO.:			EP 2015-202585 A	20151223
			EP 2016-161068 A	20160318
			EP 2016-176025 A	20160623
			EP 2016-176049 A	20160623
			EP 2016-182845 A	20160804
			WO 2016-EP82663 W	20161223

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S): CASFORMULTNS 2017:1072906
REFERENCE COUNT: 5 THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L14 ANSWER 6 OF 6 CAPLUS COPYRIGHT 2020 ACS on STN
TI Virus purification
ACCESSION NUMBER: 2017:1072907 CAPLUS Full-text
DOCUMENT NUMBER: 167:158066
TITLE: Virus purification
INVENTOR(S): Barbero Calzado, Jana; Nebenfuehr, Mario; Schlegl, Robert; Weber, Michael; Wruss, Juergen
PATENT ASSIGNEE(S): Valneva SE, Fr.
SOURCE: PCT Int. Appl., 184pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 6
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2017109223	A1	20170629	WO 2016-EP82662	20161223

W: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC,
EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,
IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS,
LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, ZA, ZM, ZW
RW: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
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ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW,
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EP 3393509	A1	20181031	EP 2016-828745	20161223

R: AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR,
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RS, SE, SI, SK, SM, TR, BA, ME

US 20180369359	A1	20181227	US 2018-16062245	20180614
PRIORITY APPLN. INFO.:			EP 2015-202585 A	20151223
			EP 2016-161068 A	20160318

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                                                EP 2016-176025       A  20160623
                                                EP 2016-176049       A  20160623
                                                EP 2016-182845       A  20160804
                                                WO 2016-EP82662      W  20161223
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
OTHER SOURCE(S):          CASFORMULTNS 2017:1072907
OS.CITING REF COUNT:      1     THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD
                                (1 CITINGS)
REFERENCE COUNT:          8     THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS
                                RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT


=> log y

     (FILE 'HOME' ENTERED AT 16:30:33 ON 15 JAN 2020)

     FILE 'MEDLINE, CAPLUS, SCISEARCH, BIOSIS, EMBASE' ENTERED AT 16:30:40 ON
     15 JAN 2020
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L2              5 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  L1 AND ZIKA
L3              5 DUP REM L2 (0 DUPLICATES REMOVED)
                  DISP L3 1-5 TI IBIB
                  E NEBENFUHR MARIO/AU
L4              0 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  !NEBENFUHR MARIO!/AU
                  E SCHLGEL ROBERT/AU
L5              0 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  !SCHLGEL ROBERT!/AU
                  E WEBER MICHAEL/AU
L6           2526 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  !WEBER MICHAEL!/AU
L7              5 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  L6 AND ZIKA
L8              5 DUP REM L7 (0 DUPLICATES REMOVED)
                  DISP L8 1-5 TI IBIB
                  E HEINDL WRUSS JURGEN/AU
L9              0 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  !HEINDL WRUSS JURGEN!/AU
                  E ZIKA
L10         33464 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  ZIKA/BI
L11         30899 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  L10 AND VIRUS
L12          4915 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  L11 AND VACCINE
L13             6 SEA FILE=MFE SPE=ON  ABB=ON  PLU=ON  L12 AND PROTAMINE
L14             6 DUP REM L13 (0 DUPLICATES REMOVED)
                  DISP L14 1-6 TI IBIB
COST IN U.S. DOLLARS                          SINCE FILE      TOTAL
                                                   ENTRY    SESSION
FULL ESTIMATED COST                              179.38     179.56

STN INTERNATIONAL LOGOFF AT 16:33:08 ON 15 JAN 2020
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/B.A.C/

DOCKET NO.: I0422.70125US00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	Barry A. Chestnut
Art Unit:	1648

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 23, 2019 Signature Melissa Mann: /Melissa Mann/

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed before the mailing of a first Office Action on the merits in the above-identified case.

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.A.C/

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the following co-pending applications that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
*16/060,054	06-07-2018	Barbero-Calzado et al.	I0422.70126US00
*15/781,825	06-06-2018	Barbero-Calzado et al.	I0422.70127US00

*A copy of this reference is not provided as the Office has waived the requirement under 37 C.F.R. § 1.98(a)(2)(iii) for submitting a copy of a cited U.S. patent application if it is scanned to the Image File Wrapper system and is available on Private PAIR .

The undersigned would like to bring to the Examiner's attention the enclosed search report or other communication from a corresponding or related International or Foreign National Application:

Serial No.	Date of Mailing	Type(s) of Communication
PCT/EP2016/082664	**04-10-2017**	**International Search Report and Written Opinion**
PCT/EP2016/082664	**06-26-2018**	**International Preliminary Report on Patentability**

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

/BARRY A CHESTNUT/ 01/15/2020

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /B.A.C/

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. I0422.70125US00 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Chelsea E. Witte-Garcia/
Chelsea E. Witte-Garcia, Ph.D.
Reg. No. 75,713
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: I0422.70125US00
Date: December 23, 2019

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	5	((("BARBERO") near3 ("CALZADO") near3 ("Jana"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:03
L2	0	((("NEBENFÃœHR") near3 ("Mario"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:03
L3	0	nebenfuhr near2 mario.inv.	USPAT	OR	OFF	2020/01/15 16:07
L4	1	((("SCHLGEL") near3 ("Robert"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:08
L5	814	((("WEBER") near3 ("Michael"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:08
L6	0	((("HEINDL-WRUSS") near3 ("JÃ¼rgen"))).INV.	US-PGPUB; USPAT; USOCR	OR	OFF	2020/01/15 16:08
L7	422	l1 or l3 or l4 or l5 or l6	USPAT	OR	OFF	2020/01/15 16:09
L8	0	"zika virus" and "protamine sulphate"	USPAT	OR	OFF	2020/01/15 16:10
L9	237	"zika virus"	USPAT	OR	OFF	2020/01/15 16:10
L10	125	l9 and vaccine	USPAT	OR	OFF	2020/01/15 16:11
L11	5	l10 and seroprotection	USPAT	OR	OFF	2020/01/15 16:11
L12	0	l11 and protamine.clm.	USPAT	OR	OFF	2020/01/15 16:18

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	B. A. Chestnut
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Dated: February 13, 2020 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
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Dear Sir:

INTRODUCTORY COMMENTS

Prior to issuance of the patent, applicant respectfully requests entry of this amendment under 37 C.F.R. § 1.312 for the above-captioned patent application.

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace the previously-filed specification with the concurrently filed Substitute Specification (clean version). The Substitute Specification contains no new matter.

REMARKS

Applicant submits herewith both marked-up and clean versions of the Substitute Specification in which the hyperlinks on pages 1 and 84 have been removed as indicated in the Notice of Allowability. No new matter has been added.

INTERVIEW SUMMARY

Applicant thanks the Examiner for the courtesy of the telephonic interview on January 13, 2020. As noted in the Applicant-Initiated Interview Summary, the Applicant clarified the sequence identifier number of the elected species.

Applicant believes no fee is due with this response. However, if a fee is due with this response, please charge our Deposit Account No. 23/2825, under Docket No. I0422.70125US00 from which the undersigned is authorized to draw.

Dated: February 13, 2020

Respectfully submitted,

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ZIKA VIRUS VACCINE

FIELD OF INVENTION

The disclosure relates to methods for the purification of Zika viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate. The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site ~~http://~~www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including

Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measures involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

A preventative vaccine against Zika virus is a pressing medical need in endemic areas and in geographical areas where the vector is spreading. Furthermore, as Zika infection has dire consequences on embryonic and fetal development, a safe and effective vaccine for women of child-bearing potential or pregnant women is needed. Vaccines administered during pregnancy must be very safe for both the mother and the developing fetus. While live attenuated viral vaccines are highly effective, they are often not considered safe enough for administration to pregnant women. In this regard, inactivated viral vaccines, which lack the ability to propagate in the vaccinated subject, are considered much safer. Development of an inactivated Zika virus vaccine for administration to at-risk patients would fill this need.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can be very efficiently separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to Zika virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely

devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Detail experimental examples to the above are provided for Zika virus.

The herein disclosed in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was unexpectedly higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, Nature doi:10.1038/nature18952.). Inactivated viruses are among the safest vaccines and especially preferred for delivery to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figure 7: Alignment (shading: % identity, aa), E-protein.

Figure 8: An example of virus particle maturation in the host cell. As observed in flaviviruses, full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID50 activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 9: An exemplary downstream virus purification process from the crude harvest to formulation of the drug substance (vaccine), a preferred embodiment of the process of the invention (**A**). A flow-chart of an exemplary virus inactivation process is shown in (**B**).

Figure 10: PS treatment results in selective removal of Zika virus aggregates and Vero HCP and LMW impurities (SEC-HPLC of 30x concentrated Zika Virus harvest day 5).

Figure 11: SEC-HPLC of individual 30x concentrated Zika harvest prior to PS treatment at different time points.

Figure 12: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment at different time points. The smaller graph indicates the observed cytopathic effect (CPE) over time.

Figure 13: Preparation of the sucrose gradient.

Figure 14: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 15: Comparison of JEV and ZikaV harvest schedules/yields.

Figure 16: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column. Both panels are from the same chromatogram. The upper graph is the complete elution profile; the lower graph is an enlargement of the ZIKAV elution peak.

Figure 17: SEC-MALLS analysis of inactivated ZikaV.

Figure 18: Cumulative particle size distribution of Zika NIV.

Figure 19: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 20: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 21: Correlation between JEV antigen content in neutralized inactivated virus (NIV) analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus.

Disclosed herein are downstream processes for purifying Zika virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for

propagation on a particular cell substrate or for a partially / fully cell substrate adapted Zika virus particle.

Aspects of the invention provide processes for the purification of infectious Zika virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the content of residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL.

In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the detection limit of HPLC, in particular below the detection limit in the final drug substance. In some embodiments, the PS content is tested by HPLC or size exclusion chromatography (SEC). For example, HPLC is validated for PS determination in JEV sucrose gradient pool samples as a routine release assay and is very sensitive (i.e., LOQ 3 µg/mL; LOD 1 µg/mL). In the current invention, PS content in in Zika virus DS samples was <LOD. In one embodiment, the HPLC assessment of PS

content can be performed on a Superdex Peptide 10/300GL column (GE: 17-5176-01) using 30% Acetonitrile, 0,1% Trifluoroacetic acid as solvent with a flow rate of 0,6 ml/min at 25°C and detection at 214 nm. A more sensitive method of measurement for residual protamine in a purified virus preparation is mass spectrometry (MS). In some embodiments, the residual PS levels in a Zika virus preparation are tested by MS or other such highly sensitive method, e.g. nuclear magnetic resonance (NMR). With this method, residual PS, as well as fragments and/or break-down products of PS, can be detected at trace amounts, such as levels as low as, for example, 10^6, 10^7 or 10^8 molecules per typical sample load. In some embodiments, the PS levels are tested in the sucrose gradient pool. In some embodiments, the PS levels are tested in the drug product. In some embodiments, the PS levels are tested in the drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of about 100 kDa.

The process according to the current invention may also comprise the use of a sucrose gradient, preferably an optimized sucrose gradient. The sucrose gradient is preferably optimized for the removal of protamine sulfate, also for the removal of immature viral particles or other viral particles which are non-infectious or host cell proteins or nucleic acids (DNA, RNA, mRNA, etc) or other host cell debris. In the current invention the optimized sucrose gradient comprises at least two, at least three, at least four layers of sucrose solutions with different densities. In one embodiment, the virus preparation to be purified is provided in a sucrose solution which has a density of about 8%, about 9%, about 10%, about 11%, about 12% sucrose (w/w), preferably about 10%. In one embodiment, one sucrose solution in the gradient has a density of about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55% sucrose (w/w), preferably about 50%. In one embodiment, one sucrose solution in the gradient has a density of about

30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40% sucrose (w/w), preferably about 35%. In one embodiment, one sucrose solution in the gradient has a density of about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20% sucrose (w/w), preferably about 15% sucrose. In a preferred embodiment, the sucrose gradient comprises three layers of sucrose solutions of about 50%, about 35% and about 15% (w/w) sucrose and the virus composition to be purified is contained in about 10% (w/w) sucrose. Because the invention provided for means to not only test for host cell DNA but also immature viral particles, the skilled person in the art is able to more precisely optimize the sucrose gradient for most efficient purification and include additional tools such as PRNT assay to monitor purification success.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may be optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the Zika virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In preferred embodiments, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian cell line. In some embodiments, said cell line is an EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the Zika virus particles obtainable by any of the processes described herein for treating and/or preventing (i.e. protecting from) a viral infection. In a preferred embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on the average for their age, sex, and ethnicity. This is a result of the brain failing to undergo proper embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has an RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the process of the invention results in an enrichment of infectious Zika virus particles from the crude harvest comprising infectious Zika virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially at least 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

A unique aspect of the current invention is the realization that know-how related to the vaccine design and purification approach used for the Japanese Encephalitis Vaccine (JEV) IXIARO® (see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) may be employed and improved upon in order to expedite the development of a Zika virus vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, was complemented by further significant improvements disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines is their greatly reduced protamine salt (SEQ ID NO: 1) content in the final drug substance facilitated by the development of an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the Vero (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or the plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be

performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation needs to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0.01 MOI (multiplicity of infection) to allow for viral production prior to passaging. In some embodiments, the Vero cells are incubated for at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. In order to minimize the adaption of Zika virus strain to Vero cells, it was found that Vero cells could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days 2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 μm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the Zika virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant", is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA. The fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of

the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in the experimental part. It was surprisingly found that the addition of a virus-containing fraction with 10% (w/w) sucrose to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) resulted in efficient separation of protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, i.e. a Zika virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agents for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphobetaine. A preferred inactivation is inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7

hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments, the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time. It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

Any of the methods or uses described herein may be for the prevention of a Zika virus infection in a subject. As used herein, the terms "prevent," "preventing" and "protection from" include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the Zika virus. As used herein, antigen(s), such as an inactivated Zika virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck, trunk, upper arms;

headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject has been previously infected with or vaccinated against Dengue virus; i.e., at risk for antibody-dependent enhancement of disease. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus antibodies). In some embodiments, the therapeutically effective amount is sufficient to provide seroprotection in a subject; i.e., to generate sufficient antigen-specific antibodies to prevent/protect from infection. In some embodiments, seroprotection is conferred on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects. In some embodiments, seroprotection is defined by a reduction in the number of Zika virus plaques by 50% or more in a plaque reduction

neutralization test (PRNT) by a 1:10 or higher dilution of sera from a vaccinated subject. In some embodiments, an effective amount of the Zika vaccine is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)). Pharmaceutical compositions are preferably manufactured under GMP

conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a

Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 71). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its entirety). In some embodiments, the adjuvant is IC31®, i.e. $KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccines to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika

virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having,", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Table 1. Overview of process buffers and stock solutions.

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.
C	25 mM Tris, 150 mM NaCl	7.4±0.2	16.5
D	1 M Tris	7.4±0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.

K	10 x PBS	7.4±0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4±0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4±0.2	1.3

Table 2. Abbreviations.

°Bx	Degrees Brix = sugar content (w/w) of an aqueous solution*
BSA	Bovine serum albumin
CC700	Capto™ Core 700
CPE	Cytopathic effect
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
ID	Inner diameter
JEV	Japanese Encephalitis virus
LAL	Limulus amebocyte lysate
LDS buffer	Lithium dodecyl sulfate sample loading buffer
LOD	Limit of detection
LOQ	Limit of quantitation
MALLS	Multiangle light scattering
mAU	Milli absorbance units
MS	Mass spectroscopy
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
PD	Process development
PFU	Plaque forming units
p.i.	Post-infection
PS	Protamine sulphate or protamine sulfate
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin
Rms radius	Root mean square radius
rMSB	Research master seed bank
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SGP	Sucrose gradient purified
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
WFI	Water for injection
ZikaV	Zika virus

*Degrees Brix (°Bx) is the sugar content of an aqueous solution. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. °Bx corresponds to the sucrose content in percent (w/w), e.g., 45 °Bx equals 45 % (w/w) sucrose.

Table A. Primers for Zika virus sequencing: lower case letters indicate bases not included in ZIKA but containing restriction sites for later cloning when needed (therefore, two Tms provided).

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
1	9320_Zika_PF_1F	SEQ ID NO: 74 ttaggatccGTTGTTGATCTGTGTGAAT	69.9	74.6	707
	9321_Zika_PF_1R	SEQ ID NO: 75 taactcgagCGTACACAACCCAAGTT	69.3	75.6	
2	9322_Zika_PF_2F	SEQ ID NO: 76 ttaggatccTCACTAGACGTGGGAGTG	70	73.9	704
	9323_Zika_PF_2R	SEQ ID NO: 77 taactcgagAAGCCATGTCYGATATTGAT	69.8	73.7	
3	9324_Zika_PF_3F	SEQ ID NO: 78 ttaggatccGCATACAGCATCAGGTG	72.3	74.5	712
	9325_Zika_PF_3R	SEQ ID NO: 79 taactcgagTGTGGAGTTCCGGTGTCT	72	76.4	
4	9326_Zika_PF_4F	SEQ ID NO: 80 ttaggatccGAATAGAGCGAARGTTGAGATA	70.9	74	712
	9327_Zika_PF_4R	SEQ ID NO: 81 taactcgAGTGGTGGGTGATCTTCTTCT	70.5	73.7	
5	9328_Zika_PF_5F	SEQ ID NO: 82 ttaggatcCAGTCACAGTGGAGGTACAGTAC	70.3	75	704
	9329_Zika_PF_5R	SEQ ID NO: 83 taactcgagCRCAGATACCATCTTCCC	71.5	77.3	
6	9330_Zika_PF_6F	SEQ ID NO: 84 ttaggatCCCTTATGTGCTTGGCCTTAG	70.7	72.7	698
	9331_Zika_PF_6R	SEQ ID NO: 85 taactcgagTCTTCAGCCTCCATGTG	70.4	76.9	
7	9332_Zika_PF_7F	SEQ ID NO: 86 ttaggatccAATGCCCACTCAAACATAGA	71.9	75	716
	9333_Zika_PF_7R	SEQ ID NO: 87 taactcgagTCATTCTCTTCTTCAGCCCTT	71	74	
8	9334_Zika_PF_8F	SEQ ID NO: 88 ttaggatccAAGGGTGATCGAGGAAT	70.9	75.2	703
	9335_Zika_PF_8R	SEQ ID NO: 89 taactcgagTTCCCTTCAGAGAGAGGAGC	71.9	73.4	
9	9336_Zika_PF_9F	SEQ ID NO: 90 ttaggatccTCTTTTGCAAACTGCGATC	71.9	75	699
	9337_Zika_PF_9R	SEQ ID NO: 91 taactcgagTCCAGCTGCAAAGGGTAT	71	74.9	
10	9338_Zika_PF_10F	SEQ ID NO: 92 ttaggatccGTGTGGACATGTACATTGA	71.4	75.8	706
	9339_Zika_PF_10R	SEQ ID NO: 93 taactcgagCCCATTGCCATAAAGTC	70.4	75.8	
11	9340_Zika_PF_11F	SEQ ID NO: 94 ttaggatccTCATACTGTGGTCCATGGA	71.6	78.1	692
	9341_Zika_PF_11R	SEQ ID NO: 95 taactcgagGCCCATCTCAACCCTTG	74	78	
12	9342_Zika_PF_12F	SEQ ID NO: 96 ttaggatccTAGAGGGCTTCCAGTGC	70.9	74	707
	9343_Zika_PF_12R	SEQ ID NO: 97 taactcgAGATACTCATCTCCAGGTTTGTTG	70.2	72.2	
13	9344_Zika_PF_13F	SEQ ID NO: 98 ttaggatccGAAAACAAAACATCAAGAGTG	70.6	75.4	726
	9345_Zika_PF_13R	SEQ ID NO: 99 taactcgagGAATCTCTCTGTCATGTGTCCT	71.9	75.6	

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
14	9346_Zika_PF_14F	SEQ ID NO: 100 ttaggatccTTGATGGCACGACCAAC	73.1	75.6	715
	9347_Zika_PF_14R	SEQ ID NO: 101 ttaggatccGTTGTTGATCTGTGTGAAT	70.8	77.9	
15	9348_Zika_PF_15F	SEQ ID NO: 102 taactcgagCAGGTCAATGTCCATTG	71.9	75.4	719
	9349_Zika_PF_15R	SEQ ID NO: 103 ttaggatccTGTTGTGTTCCTATTGCTGGT	73.9	77.2	
16	9350_Zika_PF_16F	SEQ ID NO: 104 taactcgaGTGATCAGRGCCCCAGC	72.3	75.4	703
	9351_Zika_PF_16R	SEQ ID NO: 105 ttaggatccTGCTGCCCAGAAGAGAA	72	76.3	
17	9352_Zika_PF_17F	SEQ ID NO: 106 taactcgaGCACCAACAYGGGTTCTT	73.6	76	705
	9353_Zika_PF_17R	SEQ ID NO: 107 ttaggatcCTCAAGGACGGTGTGGC	72	75.5	
18	9354_Zika_PF_18F	SEQ ID NO: 108 taactcgagCAATGATCTTCATGTTGGG	71.7	75.8	699
	9355_Zika_PF_18R	SEQ ID NO: 109 ttaggatccTATGGGGGAGGACTGGT	71	74.1	
19	9356_Zika_PF_19F	SEQ ID NO: 110 taactcGAGCCCAGAACCTTGGATC	73.3	75.5	711
	9357_Zika_PF_19R	SEQ ID NO: 111 ttaggatcCAGACCCCCAAGAAGGC	71.3	76.9	
20	9358_Zika_PF_20F	SEQ ID NO: 112 taactcgagCCCCTTTGGTCTTGTCT	71.7	75	706
	9359_Zika_PF_20R	SEQ ID NO: 113 ttaggatccAGGAAGGATGTATGCAGATG	71.9	73.9	
21	9360_Zika_PF_21F	SEQ ID NO: 114 taactcgagACATTTGCGCATATGATTTTG	70.4	75.7	709
	9361_Zika_PF_21R	SEQ ID NO: 115 ttaggatccAGGAAGGACACACAAGAGT	71.8	75	
22	9362_Zika_PF_22F	SEQ ID NO: 116 taactcgagACAGGCTGCACAGCTTT	70	79.1	581
	9363_Zika_PF_22R	SEQ ID NO: 117 ttaggatccTCTCTCATAGGGCACAGAC	74.8	81.1	

SEQUENCES

SEQ ID NO: 1
A typical form of protamine
PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 2
KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome
GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTGG
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SEQ ID NO: 3
KU497555.1 Zika virus isolate Brazil-ZKV2015, Brazil, complete genome
CCAATCTGTGAATCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTG
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SEQ ID NO: 4

KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome

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SEQ ID NO: 5

KU509998.1 Zika virus strain Haiti/1225/2014, Haiti, complete genome

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SEQ ID NO: 6
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SEQ ID NO: 7

KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome

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KU681082.3 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, complete genome

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SEQ ID NO: 9

KU707826.1 Zika virus isolate SSABR1, Brazil, complete genome

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SEQ ID NO: 10

KU744693.1 Zika virus isolate VE_Ganxian, China, complete genome

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SEQ ID NO: 11

LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome

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SEQ ID NO: 12

AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome

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CAGGTTCTTGAATGACATGGGAAAAGTTAGGAAAGACACACAGGAGTGGAAACCCTCGACTGGATGGAGCAATTGGGA
AGAAGTCCCGTTCTGCTCCCACCACTTCAACAAGCTGTACCTCAAGGATGGGAGATCCATTGTGGTCCCTTGCCGCCACCA
AGATGAACTGATTGGCCGAGCTCGCGTCTCACCAGGGGCAGGATGGAGCATCCGGGAGACTGCCTGTCTTGCAAAATCA
TATGCGCAGATGTGGCAGCTCCTTTATTTCCACAGAAGAGACCTTCGACTGATGGCTAATGCCATTTGCTCGGCTGTGCCA
GTTGACTGGGTACCAACTGGGAGAACCACCTGGTCAATCCATGGAAAGGGAGAATGGATGACCACTGAGGACATGCTCA
TGGTGTGGAATAGAGTGTGGATTGAGGAGAACGACCATATGGAGGACAAGACTCCTGTAACAAAATGGACAGACATTCC
CTATCTAGGAAAAAGGGAGGACTTATGGTGTGGATCCCTTATAGGGCACAGACCCCGCACCACTTGGGCTGAAAACATCA
AAGACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGACTATCTATCCACCCAAGTCCGCTAC
TTGGGTGAGGAAGGGTCCACACCCGGAGTGTTGTAAGCACCAATTTTAGTGTTGTCAGGCCTGCTAGTCAGCCACAGTTT
GGGGAAAGCTGTGCAGCCTGTAACCCCCCCAGGAGAAGCTGGGAAACCAAGCTCATAGTCAGGCCGAGAACGCCATGG
CACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACCCCACGCGCTTGGAAGCGCAGGAT
GGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGACTAGCTGTGAATCTCCAGCAGAGG
GACTAGTGGTTAGAGGAGACCCCCCGGAAAACGCAAAACAGCATATTGACGTGGGAAAGACCAGAGACTCCATGAGTTT
CCACCACGCTGGCCGCCAGGCACAGATCGCCGAACTTCGGCGGCCGGTGTGGGGAAATCCATGGTTTCT

SEQ ID NO: 13
KJ776791.1, Zika virus strain H/PF/2013 polyprotein gene, complete cds
AGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTC
CGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCT
GCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCTAGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCT
CATCAATAGATGGGGTTCAGTGGGGAAAAAAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATG
CTGAGAATAATCAATGCTAGGAAGGAGAAGAAGAGACGAGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGA
CCACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGC
CATATCTTTTCCAACCACATTGGGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCAT
GAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGG
GTTGTGTACGGAACCTGCCATCACAAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCAC
TAGGAAGCTGCAAACGCGGTCGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGG
ATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCAT
ATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAG
GTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACT
GTCGACATAGAGCTGGTTACAACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCGG
ACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAA
AGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTG
CATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCC
CAGCACAGTGGGATGATCGTTAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATT
CACCAAGAGCCGAAGCCACCCTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCA

GATTTGTATTACTTGACTATGAATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCAC
GCTGGGGCAGACACCGGAACTCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGG
CAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATG
GTGCAAAGGGAAGGCTGTCCTCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATAC
TCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTA
CGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTG
ATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCT
TACATTGTCATAGGAGTCGGGGAGAAGAAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTG
AAGCCACTGTGAGAGGTGCCAAGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCT
CAACTCATTGGGCAAGGGCATCCATCAAATTTTTGGAGCAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACA
AATTCTCATTGGAACGTTGCTGATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGG
GGGAGTGTTGATCTTCTTATCCACAGCTGTCTCTGCTGATGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGAT
GCGGTACAGGGGTGTTCGTCTATAACGACGTTGAAGCCTGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGA
TTGGCAGCAGCAGTCAAGCAAGCCTGGGAAGATGGTATCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTG
GAGATCAGTAGAAGGGGAGCTCAACGCAATCCTGGAAGAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAA
AACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGCCTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAAT
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AGAGCATGGAACAGCTTTCTTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGA
TTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACT
GGATTGAGAGTGAGAAGAATGACACATGGAGGCTGAAGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAA
AGTCCCACACATTGTGGACAGATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCAT
CACAATACCAGAGAGGGCTACAGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAAT
GCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAG
GGTGATCGAGGAATGGTGCTGCAGGGAGTGCACAATGCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGA
ATGGAGATAAGGCCCAGGAAAGAACCAGAAAGTAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGG
ATCACTTCTCCCTTGGAGTGCTTGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATC
ATAAGCACATCGATGGCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTT
GATGGGTGCCACCTTCGCGGAAATGAACACTGGAGGAGATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGA
CCAGCGTTGCTGGTATCTTTCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTT
TGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGATGGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAG
CGATGGTTGTTCCACGCACTGATAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTG
TGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAA
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AAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTC
GCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGA
GTGTGGACATGTACATTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCT
CGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAG
GTGGTCCTGATGACCATCTGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGAC
TGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTA
CAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGT
GGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATC
TGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCC
CGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCT
GGATTACCCAGCAGGAACTTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTC
GTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGC
CTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAA
ATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGA
AGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAAT
GTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCAC
TTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCAT
GACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAG
AGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAAC
GGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAAACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGT
TCCAGAAAACAAAACATCAAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGAC
CGTGTCATAGATTCCAGGAGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGT

CACACATGCCAGCGCTGCCCAGAGGAGGGGGCGCATAGGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGA
GGTGGGTGCGCAGAGACTGACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGA
TGGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAG
CAAAGGAAGACCTTTGTGGAACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAAT
AACCTACACAGATAGAAGATGGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTG
TGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGA
AGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACA
CATGACAGAGAGATTCCAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAA
GCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTT
TTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATG
TGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAG
CCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTAC
CGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAAC
CATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCA
GCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGT
ATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC
CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGC
GTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACAC
AATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGT
CGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGA
ACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTA
CACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGG
CCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCC
CGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTG
GAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCA
CCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTA
TGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTG
ACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTT
GAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCA
GCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCG
AAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGA
AATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCAT
TGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGG
GCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAA
ACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAG
GAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGA
AAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATT
AGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGT
GGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGA
AACAAGGGGAATTTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGA
AGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGAGAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACA
AAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCAT
TGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGA
CATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGC
AACTCATTCGGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGAC
CAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATT
GATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAAACCCT
CAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCC
ATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGG
AGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCA
ATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAATGG
ATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACCACATGGAAGACAAGACCCCA
GTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGA

CTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTC
AGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTA
TAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACC
CCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGAT
CAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAG

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13 or 72. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13 or 72.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 14
isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 15
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
RDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 16
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXXXXXXXNR
AEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 17
ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMXXXXXGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 18
ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 19
ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 20
isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 21
isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 22
isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVE
FKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA

FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 23
MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 24
LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 25
isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 26
ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 27
AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 28
MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 29
MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 30
KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 31
isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 32
CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 33
Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN

RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 34
isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 35
KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 36
KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 37
isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 38
BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 39
MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 40
KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 41
Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 42
KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 43
isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 44
isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 45
PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 46
BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 47
H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 48
PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 49
KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF

EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 50
ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 51
103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 52
isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 53
KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 54
isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 55

isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 56
isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 57
isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 58
isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 59
isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNGTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 60
isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 61
ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 62
KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 63
isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 64
isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCPTQGEAYLD
KQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENR
AKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFK
DAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVE
VQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 65
isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 66
KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 67
isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 68
ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E.
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCPTQGEPSL
DKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 69
MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 70
5'-$(dIdC)_{13}$-3'
dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC

SEQ ID NO: 71
KLK peptide
KLKLLLLLKLK

SEQ ID NO: 72
ZIKV Sequence H/PF/2013 as sequenced
CAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTT
TCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTG
AGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCT
AGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAAAAGAGGCTA

TGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGAAGGAGAAGAAGAGAC
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TGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTT
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ATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTG
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CATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACTGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTC
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TGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAA
GGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCT
GGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAA
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AAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAG
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GCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAAATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGA
TCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCA
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TCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGTATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGG
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SEQ ID NO: 73

AHZ13508.1, Zika virus polyprotein from Polynesian outbreak (H/PF/2013)

MKNPKKKSGGFRIVNMLKRGVARVSPFGGLKRLPAGLLLGHGPIRMVLAILAFLRFTAIKPSLGLINRWGSVGKKEAMEIIKKFKK
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SGAKSNTIKSVSTTSQLLLGRMDGPRRPVKYEEDVNLGSGTRAVVSCAEAPNMKIIGNRIERIRSEHAETWFFDENHPYRTWAY
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SEQ ID NO: 74
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SEQ ID NO: 75
9321_Zika_PF_1R
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SEQ ID NO: 76
9322_Zika_PF_2F
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SEQ ID NO: 77
9323_Zika_PF_2R
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SEQ ID NO: 78
9324_Zika_PF_3F
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SEQ ID NO: 79
9325_Zika_PF_3R
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SEQ ID NO: 80
9326_Zika_PF_4F
ttaggatccGAATAGAGCGAARGTTGAGATA

SEQ ID NO: 81
9327_Zika_PF_4R
taactcgAGTGGTGGGTGATCTTCTTCT

SEQ ID NO: 82
9328_Zika_PF_5F
ttaggatcCAGTCACAGTGGAGGTACAGTAC

SEQ ID NO: 83
9329_Zika_PF_5R
taactcgagCRCAGATACCATCTTCCC

SEQ ID NO: 84
9330_Zika_PF_6F

ttaggatCCCTTATGTGCTTGGCCTTAG

SEQ ID NO: 85
9331_Zika_PF_6R
taactcgagTCTTCAGCCTCCATGTG

SEQ ID NO: 86
9332_Zika_PF_7F
ttaggatccAATGCCCACTCAAACATAGA

SEQ ID NO: 87
9333_Zika_PF_7R
taactcgagTCATTCTCTTCTTCAGCCCTT

SEQ ID NO: 88
9334_Zika_PF_8F
ttaggatccAAGGGTGATCGAGGAAT

SEQ ID NO: 89
9335_Zika_PF_8R
taactcgagTTCCCTTCAGAGAGAGGAGC

SEQ ID NO: 90
9336_Zika_PF_9F
ttaggatccTCTTTTGCAAACTGCGATC

SEQ ID NO: 91
9337_Zika_PF_9R
taactcgagTCCAGCTGCAAAGGGTAT

SEQ ID NO: 92
9338_Zika_PF_10F
ttaggatccGTGTGGACATGTACATTGA

SEQ ID NO: 93
9339_Zika_PF_10R
taactcgagCCCATTGCCATAAAGTC

SEQ ID NO: 94
9340_Zika_PF_11F
ttaggatccTCATACTGTGGTCCATGGA

SEQ ID NO: 95
9341_Zika_PF_11R
taactcgagGCCCATCTCAACCCTTG

SEQ ID NO: 96
9342_Zika_PF_12F
ttaggatccTAGAGGGCTTCCAGTGC

SEQ ID NO: 97
9343_Zika_PF_12R
taactcgAGATACTCATCTCCAGGTTTGTTG

SEQ ID NO: 98

9344_Zika_PF_13F
ttaggatccGAAAACAAAACATCAAGAGTG

SEQ ID NO: 99
9345_Zika_PF_13R
taactcgagGAATCTCTCTGTCATGTGTCCT

SEQ ID NO: 100
9346_Zika_PF_14F
ttaggatccTTGATGGCACGACCAAC

SEQ ID NO: 101
9347_Zika_PF_14R
ttaggatccGTTGTTGATCTGTGTGAAT

SEQ ID NO: 102
9348_Zika_PF_15F
taactcgagCAGGTCAATGTCCATTG

SEQ ID NO: 103
9349_Zika_PF_15R
ttaggatccTGTTGTGTTCCTATTGCTGGT

SEQ ID NO: 104
9350_Zika_PF_16F
taactcgaGTGATCAGRGCCCCAGC

SEQ ID NO: 105
9351_Zika_PF_16R
ttaggatccTGCTGCCCAGAAGAGAA

SEQ ID NO: 106
9352_Zika_PF_17F
taactcgaGCACCAACAYGGGTTCTT

SEQ ID NO: 107
9353_Zika_PF_17R
ttaggatcCTCAAGGACGGTGTGGC

SEQ ID NO: 108
9354_Zika_PF_18F
taactcgagCAATGATCTTCATGTTGGG

SEQ ID NO: 109
9355_Zika_PF_18R
ttaggatccTATGGGGGAGGACTGGT

SEQ ID NO: 110
9356_Zika_PF_19F
taactcGAGCCCAGAACCTTGGATC

SEQ ID NO: 111
9357_Zika_PF_19R
ttaggatcCAGACCCCCAAGAAGGC

SEQ ID NO: 112
9358_Zika_PF_20F
taactcgagCCCCTTTGGTCTTGTCT

SEQ ID NO: 113
9359_Zika_PF_20R
ttaggatccAGGAAGGATGTATGCAGATG

SEQ ID NO: 114
9360_Zika_PF_21F
taactcgagACATTTGCGCATATGATTTTG

SEQ ID NO: 115
9361_Zika_PF_21R
ttaggatccAGGAAGGACACACAAGAGT

SEQ ID NO: 116
9362_Zika_PF_22F
taactcgagACAGGCTGCACAGCTTT

SEQ ID NO: 117
9363_Zika_PF_22R
ttaggatccTCTCTCATAGGGCACAGAC

In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NOs: 14-69 or 72. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69 or 72.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. MoI. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Dr., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977 and Altschul et al., J. MoI. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were adapted to ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 9A and B). Briefly, the unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Zika Virus. As shown in Figure 10, non-infectious virus particle aggregates, HCP and other LMW impurities were removed by PS precipitation as shown by removal of aggregate shoulder in SEC-HPLC and no loss of infectious virus titer by PS treatment. Further optimization of the Zika purification protocol is provided below.

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):
- 5% CO_2, 35°C, MEM+2mM L-Glutamine + 10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01

- Virus Production without serum
- 5% CO_2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (accession number KJ776791.1, also SEQ ID NO: 13 herein) from French Polynesia. A sample was obtained from the European Virus Archive (EVAg; Ref-SKU: 001v-EVA1545). Based on this material, a research master seed bank (rMSB) was prepared on Vero cells as the cell substrate and the genomic sequence was checked by sequencing. Because the genomic sequence at the 5´and 3´flanking sequences of Zika virus strain H/PF/2013 was unknown, primers for sequencing were designed in those regions based on other Zika virus strains whereas the internal primers were designed from the published sequence (SEQ ID NOs: 74 to 117, see also Table A). The sequence obtained from the rMSB by use of these primers is provided by SEQ ID NO: 72. There was 100% overlap of the sequence with the published sequence of Zika Virus Strain H/PF/2013 (SEQ ID NO: 13). However, we sequenced additional regions 5´ (an additional 40 bp) and 3' (an additional 160 bp) represented in SEQ ID NO: 72. In a preferred embodiment, the Zika virus of the invention comprises SEQ ID NO: 72. The genomic RNA is somewhat longer than the sequence according to SEQ ID NO: 72 (perhaps an additional 200 bp). Additionally, a Zika virus adapted to a host cell such as e.g. Vero cells may be expected to contain one or more mutations. For these reasons, the Zika virus of the current invention comprises the sequence of SEQ ID NO: 72 or, preferably, a sequence with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 72. Furthermore, because the viral genome is likely to contain even further flanking regions to SEQ ID NO: 72; in one embodiment, the Zika virus of the invention contains the sequence of SEQ ID NO: 72 and optionally further comprises extensions at the 5' and/or 3' ends of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120 or at least 130 nucleotides. In a preferred embodiment, the Zika virus comprises at least the coding sequence for the entire

polyprotein of Zika Virus Strain H/PF/2013 of the invention i.e. the amino acid sequence of SEQ ID NO: 73 or a polyprotein with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 73. Furthermore, the Zika virus comprises at least the coding sequence for the E-protein of Zika Virus Strain H/PF/2013 of the invention SEQ ID NO: 47 or an E-protein thereof with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 47.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in a standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and to reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles were infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200 mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significantly higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with the day 3 harvest, the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest.

Table 3: The calculated titers per plaque assay are summarized in the list below.

	Log 10 PFU/mL
sample day2	7.02
harvest day 3	6.66
harvest day 5	6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 15. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22°C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 11 for SEC-HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 20 for SEC-HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 21). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

Samples of individual 30x concentrated harvests days 2, 3, 5 and 7 were analysed before (Figure 11) and after PS (Figure 12) treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for

determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 4. The virus peak recovery by SEC-HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 4: ZikaV recovery by SEC-HPLC and plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x + PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x + PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles. Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus particles from residual contaminants as demonstrated for ChikV (Fig. 15D). The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 5.

Table 5: Individual layers/volumes for a centrifugation in bottle.

Solution	**Volume (mL)**
PS treated harvest in 10% sucrose (L)	40
15% sucrose (J)	15
35% sucrose (I)	15
50% sucrose (H)	20
Total volume	90

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 14. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled rotor. (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers.) The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no brake/deceleration activated. In case a different centrifuge system with a different rotor is used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle (see Figure 14).

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL)

and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 14. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51 mL in a PETG container. A volume of 1% formaldehyde (10,000 ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200 ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25°C) for a minimum of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criterion for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution was monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to

unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152 µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. Briefly, a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 21.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gently mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 6).

Table 6: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	JEV	Zika
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2 µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565.

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 16. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 17) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6 nm and ~49 nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 18).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Table 7: Active ZikaV pfus were quantified by plaque assay throughout the process.

Sample	Pfu/mL
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$
Harvest day 5 (filtered)	$1.5x10^8$
Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD

Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2 mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly, the Benzonase treated samples did not yield any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell protein was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well was seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5% CO_2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicate. The target viruses (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were diluted to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were

incubated at 35°C with 5% CO_2 for 1 hour. The cell culture medium was aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plates were rocked back and forth 5 times before incubating for 2 hours at 35°C with 5% CO_2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5% CO_2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 19 and 20, respectively). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Another neutralization assay was performed using the microneutralization assay as described by Larocca, et al. (2016, Nature doi:10.1038/nature18952). It was found that the inactivated Zika virus of the current invention had an MN50 (microneutralization) titer of 90 at 1 µg of inactivated purified virus.

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, ~~http://~~dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by

administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on Vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV vaccine. The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

The in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-

protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, supra.). Inactivated viruses are among the safest vaccines and especially preferred for deliver to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

A4. The vaccine of any one of A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of A1-A9, further comprising an adjuvant.

A11. The vaccine of A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

A15. The vaccine of any one of A1-A14, further comprising one or more pharmaceutically acceptable excipient.

A16. The vaccine of any one of A1-A15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 μg/mL, especially below 100 ng/mL.

A17. The vaccine of A16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

B1. A kit comprising a Zika virus vaccine of any one of A1-A15.

B2. The kit of B1, further comprising a second vaccine.

B3. The kit of B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of A1-A15 to a subject in need thereof.

C2. The method of C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of A1-A15 for the treatment and/or prevention of a Zika virus infection.

E2. The use of E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment or prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of A1-A15.

F2. The pharmaceutical composition of F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

G1. Use of an optimized sucrose gradient centrifugation for removal of protamine sulphate from purified infectious Zika virus particles.

G2. The use according to G1, wherein said optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three further layers of sucrose solutions with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G3. A process of purification of infectious Zika virus particles, comprising the steps of:

a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

G4. The process of G3, wherein said optimized sucrose density gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G5. The process of any one of G3 to G4, additionally comprising a further purification step of: (d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from

entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

G6. The process of any of G3 to G5, wherein the residual host cell DNA content of the Zika virus preparation (c) is less than 10 ng/mL and the residual host cell protein content of the final virus preparation (c) is less than 100 ng/mL.

G7. The process of any of G3 to G6, wherein said crude harvest (a) comprising Zika virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

G8. The process of G7, wherein the one or more pre-purification step(s) comprises

a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

b) digestion of host cell genomic DNA by enzymatic treatment; and/or

c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

G9. The process of any one of G3 to G8, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

G10. The process of any one of G3 to G9, wherein the enrichment of infectious Zika virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

G11. The process of any one of G7 to G10, wherein the one or more pre-purification step(s) prior to step (b) of any of G8 to G11 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

G12. The process of any one of G3 to G11, wherein the residual impurity of the Zika virus preparation (c) is less than 10%.

G13. The process of any one of G3 to G12, wherein the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a

PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

G14. The process of G13, wherein said cell line is a Vero cell line.

G15. The process of any one of G3 to G14, wherein said infectious Zika virus particle is an infectious virus particle that is a live virus, a live attenuated virus, a chimeric virus, a modified live virus, or a recombinant live virus.

G16. The process of any one of G3 to G15, wherein said Zika virus is preferably a strain of the Asian lineage.

G17. The process of any one of G3 to G16, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

G18. Use of the process according to any one of G3 to G17 for manufacturing a composition for immunization against a virus infection.

G19. The use according to G18, wherein said virus infection is an infection caused by a Zika virus.

Q1. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

Q2. The process of Q2, wherein the virus particles are from Zika virus.

Q3. The process of Q1 or Q2, additionally comprising the step of:

(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

Q4. The process of any of Q1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

Q5. The process of any of Q1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

Q6. The process of Q5, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

Q7. The process of any one of Q1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

Q8. The process of any one of Q1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

Q9. The process of any one of Q5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of Q5 to 8 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

Q10. The process of any one of Q1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

Q11. The process of any one of Q1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

Q12. The process of Q11, wherein said cell line is a Vero cell line.

Q13. The process of any one of Q1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

Q14. The process of any one of Q1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

Q15. The process of any one of Q1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

Q16. Use of the process according to any one of Q1 to 15 for manufacturing a composition for immunization against a virus infection.

Q17. The use according to Q16, wherein the composition for immunization against a virus infection is an infection caused by Zika virus.

Q18. A composition comprising the virus particles obtainable or obtained by the process of any one of Q1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

Q19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

Q20. The Zika virus vaccine of Q19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

Q21. The vaccine of Q19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

Q22. The vaccine of any one of Q19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69 or 72, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 or 72 and able to pack a virulent Zika virus.

Q23. The vaccine of any one of Q19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

Q24. The vaccine of Q23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

Q25. The vaccine of Q24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

Q26. The vaccine of any one of Q19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

Q27. The vaccine of Q26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

Q28. The vaccine of Q27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

Q29. The vaccine of Q28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

Q30. The vaccine of any one of Q27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

Q31. The vaccine of any one of Q19 to 30, further comprising an adjuvant.

Q32. The vaccine of Q31, wherein the adjuvant is an aluminum salt adjuvant.

Q33. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

Q34. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

Q35. The vaccine of any one of Q19 to 34, further comprising one or more pharmaceutically acceptable excipient.

R1. Use of protamine, preferably a protamine salt, to separate infectious and non-infectious Zika virus particles, host cell proteins and/or undefined low molecular weight materials.

R2. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b), wherein the enrichment of infectious virus particles in the virus preparation (b) relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R3. The use of R1 or the process of R2, wherein the virus particles are from Zika virus.

R4. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by one or more size exclusion methods such as (i) a sucrose density gradient centrifugation, (ii) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles, and/or (iii) size exclusion chromatography to obtain a virus preparation (c) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (c) is less than 100 ng/mL and the residual host cell protein and the residual aggregates of infectious virus particles of the final virus preparation (c) is less than 1 μg/mL.

R5. The process of R4, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

R6. The process of any of R2 to 5, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

R7. The process of R6, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

R8. The process of any one of R2 to 7, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.

R9. The process of any one of R2 to 8, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R10. The process of any one of R6 to 9, wherein the one or more pre-purification step(s) prior to step (b) of any of R6 to 9 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

R11. The process of any one of R2 to 10, wherein the residual impurity of the virus preparation (c) is less than 10%.

R12. The process of any one of R2 to 11, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

R13. The process of R12, wherein said cell line is a Vero cell line.

R14. The process of any one of R2 to 13, wherein the Zika virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

R15. The process of any one of R2 to 14, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

R16. The process of any one of R2 to 15, wherein said process resulting in final virus preparation (c) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

R17. Use of the process according to any one of R1 to 16 for manufacturing a composition for immunization against a virus infection.

R18. The use according to R17, wherein the composition for immunization against a virus infection is an infection caused by a Zika virus.

R19. A composition comprising the virus particles obtainable or obtained by the process of any one of R2 to 16 for treating and/or preventing an infection.

ZIKA VIRUS VACCINE

FIELD OF INVENTION

The disclosure relates to methods for the purification of Zika viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate. The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including

Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measures involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

A preventative vaccine against Zika virus is a pressing medical need in endemic areas and in geographical areas where the vector is spreading. Furthermore, as Zika infection has dire consequences on embryonic and fetal development, a safe and effective vaccine for women of child-bearing potential or pregnant women is needed. Vaccines administered during pregnancy must be very safe for both the mother and the developing fetus. While live attenuated viral vaccines are highly effective, they are often not considered safe enough for administration to pregnant women. In this regard, inactivated viral vaccines, which lack the ability to propagate in the vaccinated subject, are considered much safer. Development of an inactivated Zika virus vaccine for administration to at-risk patients would fill this need.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can be very efficiently separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to Zika virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely

devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Detail experimental examples to the above are provided for Zika virus.

The herein disclosed in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was unexpectedly higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, Nature doi:10.1038/nature18952.). Inactivated viruses are among the safest vaccines and especially preferred for delivery to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figure 7: Alignment (shading: % identity, aa), E-protein.

Figure 8: An example of virus particle maturation in the host cell. As observed in flaviviruses, full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID50 activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 9: An exemplary downstream virus purification process from the crude harvest to formulation of the drug substance (vaccine), a preferred embodiment of the process of the invention (**A**). A flow-chart of an exemplary virus inactivation process is shown in (**B**).

Figure 10: PS treatment results in selective removal of Zika virus aggregates and Vero HCP and LMW impurities (SEC-HPLC of 30x concentrated Zika Virus harvest day 5).

Figure 11: SEC-HPLC of individual 30x concentrated Zika harvest prior to PS treatment at different time points.

Figure 12: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment at different time points. The smaller graph indicates the observed cytopathic effect (CPE) over time.

Figure 13: Preparation of the sucrose gradient.

Figure 14: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 15: Comparison of JEV and ZikaV harvest schedules/yields.

Figure 16: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column. Both panels are from the same chromatogram. The upper graph is the complete elution profile; the lower graph is an enlargement of the ZIKAV elution peak.

Figure 17: SEC-MALLS analysis of inactivated ZikaV.

Figure 18: Cumulative particle size distribution of Zika NIV.

Figure 19: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 20: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 21: Correlation between JEV antigen content in neutralized inactivated virus (NIV) analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus.

Disclosed herein are downstream processes for purifying Zika virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for

propagation on a particular cell substrate or for a partially / fully cell substrate adapted Zika virus particle.

Aspects of the invention provide processes for the purification of infectious Zika virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the content of residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL.

In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the detection limit of HPLC, in particular below the detection limit in the final drug substance. In some embodiments, the PS content is tested by HPLC or size exclusion chromatography (SEC). For example, HPLC is validated for PS determination in JEV sucrose gradient pool samples as a routine release assay and is very sensitive (i.e., LOQ 3 µg/mL; LOD 1 µg/mL). In the current invention, PS content in in Zika virus DS samples was <LOD. In one embodiment, the HPLC assessment of PS

content can be performed on a Superdex Peptide 10/300GL column (GE: 17-5176-01) using 30% Acetonitrile, 0,1% Trifluoroacetic acid as solvent with a flow rate of 0,6 ml/min at 25°C and detection at 214 nm. A more sensitive method of measurement for residual protamine in a purified virus preparation is mass spectrometry (MS). In some embodiments, the residual PS levels in a Zika virus preparation are tested by MS or other such highly sensitive method, e.g. nuclear magnetic resonance (NMR). With this method, residual PS, as well as fragments and/or break-down products of PS, can be detected at trace amounts, such as levels as low as, for example, 10^6, 10^7 or 10^8 molecules per typical sample load. In some embodiments, the PS levels are tested in the sucrose gradient pool. In some embodiments, the PS levels are tested in the drug product. In some embodiments, the PS levels are tested in the drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of about 100 kDa.

The process according to the current invention may also comprise the use of a sucrose gradient, preferably an optimized sucrose gradient. The sucrose gradient is preferably optimized for the removal of protamine sulfate, also for the removal of immature viral particles or other viral particles which are non-infectious or host cell proteins or nucleic acids (DNA, RNA, mRNA, etc) or other host cell debris. In the current invention the optimized sucrose gradient comprises at least two, at least three, at least four layers of sucrose solutions with different densities. In one embodiment, the virus preparation to be purified is provided in a sucrose solution which has a density of about 8%, about 9%, about 10%, about 11%, about 12% sucrose (w/w), preferably about 10%. In one embodiment, one sucrose solution in the gradient has a density of about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55% sucrose (w/w), preferably about 50%. In one embodiment, one sucrose solution in the gradient has a density of about

30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40% sucrose (w/w), preferably about 35%. In one embodiment, one sucrose solution in the gradient has a density of about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20% sucrose (w/w), preferably about 15% sucrose. In a preferred embodiment, the sucrose gradient comprises three layers of sucrose solutions of about 50%, about 35% and about 15% (w/w) sucrose and the virus composition to be purified is contained in about 10% (w/w) sucrose. Because the invention provided for means to not only test for host cell DNA but also immature viral particles, the skilled person in the art is able to more precisely optimize the sucrose gradient for most efficient purification and include additional tools such as PRNT assay to monitor purification success.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may be optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the Zika virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In preferred embodiments, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian cell line. In some embodiments, said cell line is an EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the Zika virus particles obtainable by any of the processes described herein for treating and/or preventing (i.e. protecting from) a viral infection. In a preferred embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on the average for their age, sex, and ethnicity. This is a result of the brain failing to undergo proper embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has an RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the process of the invention results in an enrichment of infectious Zika virus particles from the crude harvest comprising infectious Zika virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially at least 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

A unique aspect of the current invention is the realization that know-how related to the vaccine design and purification approach used for the Japanese Encephalitis Vaccine (JEV) IXIARO® (see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) may be employed and improved upon in order to expedite the development of a Zika virus vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, was complemented by further significant improvements disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines is their greatly reduced protamine salt (SEQ ID NO: 1) content in the final drug substance facilitated by the development of an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the Vero (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or the plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be

performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation needs to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0.01 MOI (multiplicity of infection) to allow for viral production prior to passaging. In some embodiments, the Vero cells are incubated for at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. In order to minimize the adaption of Zika virus strain to Vero cells, it was found that Vero cells could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days 2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 μm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the Zika virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant", is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA. The fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of

the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in the experimental part. It was surprisingly found that the addition of a virus-containing fraction with 10% (w/w) sucrose to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) resulted in efficient separation of protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, i.e. a Zika virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agents for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphobetaine. A preferred inactivation is inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7

hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments, the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time. It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

Any of the methods or uses described herein may be for the prevention of a Zika virus infection in a subject. As used herein, the terms "prevent," "preventing" and "protection from" include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the Zika virus. As used herein, antigen(s), such as an inactivated Zika virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck, trunk, upper arms;

headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject has been previously infected with or vaccinated against Dengue virus; i.e., at risk for antibody-dependent enhancement of disease. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus antibodies). In some embodiments, the therapeutically effective amount is sufficient to provide seroprotection in a subject; i.e., to generate sufficient antigen-specific antibodies to prevent/protect from infection. In some embodiments, seroprotection is conferred on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects. In some embodiments, seroprotection is defined by a reduction in the number of Zika virus plaques by 50% or more in a plaque reduction

neutralization test (PRNT) by a 1:10 or higher dilution of sera from a vaccinated subject. In some embodiments, an effective amount of the Zika vaccine is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)). Pharmaceutical compositions are preferably manufactured under GMP

conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a

Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 71). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its entirety). In some embodiments, the adjuvant is IC31®, i.e. $KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccines to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika

virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having,", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Table 1. Overview of process buffers and stock solutions.

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.
C	25 mM Tris, 150 mM NaCl	7.4±0.2	16.5
D	1 M Tris	7.4±0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.

K	10 x PBS	7.4±0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4±0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4±0.2	1.3

Table 2. Abbreviations.

°Bx	Degrees Brix = sugar content (w/w) of an aqueous solution*
BSA	Bovine serum albumin
CC700	Capto™ Core 700
CPE	Cytopathic effect
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
ID	Inner diameter
JEV	Japanese Encephalitis virus
LAL	Limulus amebocyte lysate
LDS buffer	Lithium dodecyl sulfate sample loading buffer
LOD	Limit of detection
LOQ	Limit of quantitation
MALLS	Multiangle light scattering
mAU	Milli absorbance units
MS	Mass spectroscopy
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
PD	Process development
PFU	Plaque forming units
p.i.	Post-infection
PS	Protamine sulphate or protamine sulfate
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin
Rms radius	Root mean square radius
rMSB	Research master seed bank
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SGP	Sucrose gradient purified
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
WFI	Water for injection
ZikaV	Zika virus

*Degrees Brix (°Bx) is the sugar content of an aqueous solution. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. °Bx corresponds to the sucrose content in percent (w/w), e.g., 45 °Bx equals 45 % (w/w) sucrose.

Table A. Primers for Zika virus sequencing: lower case letters indicate bases not included in ZIKA but containing restriction sites for later cloning when needed (therefore, two Tms provided).

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
1	9320_Zika_PF_1F	SEQ ID NO: 74 ttaggatccGTTGTTGATCTGTGTGAAT	69.9	74.6	707
	9321_Zika_PF_1R	SEQ ID NO: 75 taactcgagCGTACACAACCCAAGTT	69.3	75.6	
2	9322_Zika_PF_2F	SEQ ID NO: 76 ttaggatccTCACTAGACGTGGGAGTG	70	73.9	704
	9323_Zika_PF_2R	SEQ ID NO: 77 taactcgagAAGCCATGTCYGATATTGAT	69.8	73.7	
3	9324_Zika_PF_3F	SEQ ID NO: 78 ttaggatccGCATACAGCATCAGGTG	72.3	74.5	712
	9325_Zika_PF_3R	SEQ ID NO: 79 taactcgagTGTGGAGTTCCGGTGTCT	72	76.4	
4	9326_Zika_PF_4F	SEQ ID NO: 80 ttaggatccGAATAGAGCGAARGTTGAGATA	70.9	74	712
	9327_Zika_PF_4R	SEQ ID NO: 81 taactcgAGTGGTGGGTGATCTTCTTCT	70.5	73.7	
5	9328_Zika_PF_5F	SEQ ID NO: 82 ttaggatcCAGTCACAGTGGAGGTACAGTAC	70.3	75	704
	9329_Zika_PF_5R	SEQ ID NO: 83 taactcgagCRCAGATACCATCTTCCC	71.5	77.3	
6	9330_Zika_PF_6F	SEQ ID NO: 84 ttaggatCCCTTATGTGCTTGGCCTTAG	70.7	72.7	698
	9331_Zika_PF_6R	SEQ ID NO: 85 taactcgagTCTTCAGCCTCCATGTG	70.4	76.9	
7	9332_Zika_PF_7F	SEQ ID NO: 86 ttaggatccAATGCCCACTCAAACATAGA	71.9	75	716
	9333_Zika_PF_7R	SEQ ID NO: 87 taactcgagTCATTCTCTTCTTCAGCCCTT	71	74	
8	9334_Zika_PF_8F	SEQ ID NO: 88 ttaggatccAAGGGTGATCGAGGAAT	70.9	75.2	703
	9335_Zika_PF_8R	SEQ ID NO: 89 taactcgagTTCCCTTCAGAGAGAGGAGC	71.9	73.4	
9	9336_Zika_PF_9F	SEQ ID NO: 90 ttaggatccTCTTTTGCAAACTGCGATC	71.9	75	699
	9337_Zika_PF_9R	SEQ ID NO: 91 taactcgagTCCAGCTGCAAAGGGTAT	71	74.9	
10	9338_Zika_PF_10F	SEQ ID NO: 92 ttaggatccGTGTGGACATGTACATTGA	71.4	75.8	706
	9339_Zika_PF_10R	SEQ ID NO: 93 taactcgagCCCATTGCCATAAAGTC	70.4	75.8	
11	9340_Zika_PF_11F	SEQ ID NO: 94 ttaggatccTCATACTGTGGTCCATGGA	71.6	78.1	692
	9341_Zika_PF_11R	SEQ ID NO: 95 taactcgagGCCCATCTCAACCCTTG	74	78	
12	9342_Zika_PF_12F	SEQ ID NO: 96 ttaggatccTAGAGGGCTTCCAGTGC	70.9	74	707
	9343_Zika_PF_12R	SEQ ID NO: 97 taactcgAGATACTCATCTCCAGGTTTGTTG	70.2	72.2	
13	9344_Zika_PF_13F	SEQ ID NO: 98 ttaggatccGAAAACAAAACATCAAGAGTG	70.6	75.4	726
	9345_Zika_PF_13R	SEQ ID NO: 99 taactcgagGAATCTCTCTGTCATGTGTCCT	71.9	75.6	

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
14	9346_Zika_PF_14F	SEQ ID NO: 100 ttaggatccTTGATGGCACGACCAAC	73.1	75.6	715
	9347_Zika_PF_14R	SEQ ID NO: 101 ttaggatccGTTGTTGATCTGTGTGAAT	70.8	77.9	
15	9348_Zika_PF_15F	SEQ ID NO: 102 taactcgagCAGGTCAATGTCCATTG	71.9	75.4	719
	9349_Zika_PF_15R	SEQ ID NO: 103 ttaggatccTGTTGTGTTCCTATTGCTGGT	73.9	77.2	
16	9350_Zika_PF_16F	SEQ ID NO: 104 taactcgaGTGATCAGRGCCCCAGC	72.3	75.4	703
	9351_Zika_PF_16R	SEQ ID NO: 105 ttaggatccTGCTGCCCAGAAGAGAA	72	76.3	
17	9352_Zika_PF_17F	SEQ ID NO: 106 taactcgaGCACCAACAYGGGTTCTT	73.6	76	705
	9353_Zika_PF_17R	SEQ ID NO: 107 ttaggatcCTCAAGGACGGTGTGGC	72	75.5	
18	9354_Zika_PF_18F	SEQ ID NO: 108 taactcgagCAATGATCTTCATGTTGGG	71.7	75.8	699
	9355_Zika_PF_18R	SEQ ID NO: 109 ttaggatccTATGGGGGAGGACTGGT	71	74.1	
19	9356_Zika_PF_19F	SEQ ID NO: 110 taactcGAGCCCAGAACCTTGGATC	73.3	75.5	711
	9357_Zika_PF_19R	SEQ ID NO: 111 ttaggatcCAGACCCCCAAGAAGGC	71.3	76.9	
20	9358_Zika_PF_20F	SEQ ID NO: 112 taactcgagCCCCTTTGGTCTTGTCT	71.7	75	706
	9359_Zika_PF_20R	SEQ ID NO: 113 ttaggatccAGGAAGGATGTATGCAGATG	71.9	73.9	
21	9360_Zika_PF_21F	SEQ ID NO: 114 taactcgagACATTTGCGCATATGATTTTG	70.4	75.7	709
	9361_Zika_PF_21R	SEQ ID NO: 115 ttaggatccAGGAAGGACACACAAGAGT	71.8	75	
22	9362_Zika_PF_22F	SEQ ID NO: 116 taactcgagACAGGCTGCACAGCTTT	70	79.1	581
	9363_Zika_PF_22R	SEQ ID NO: 117 ttaggatccTCTCTCATAGGGCACAGAC	74.8	81.1	

SEQUENCES

SEQ ID NO: 1
A typical form of protamine
PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 2
KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome
GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTGG
ATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACG
CGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGG
ATGGTCTTGGCAATTCTAGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTG

GGGAAAAAAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGA
AGGAGAAGAAGAGACGGGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGG
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SEQ ID NO: 4

KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome

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SEQ ID NO: 5

KU509998.1 Zika virus strain Haiti/1225/2014, Haiti, complete genome

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KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome

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KU681082.3 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, complete genome

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SEQ ID NO: 9

KU707826.1 Zika virus isolate SSABR1, Brazil, complete genome

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KU744693.1 Zika virus isolate VE_Ganxian, China, complete genome

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SEQ ID NO: 11

LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome

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SEQ ID NO: 12

AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome

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SEQ ID NO: 13

KJ776791.1, Zika virus strain H/PF/2013 polyprotein gene, complete cds

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CCACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGC
CATATCTTTTCCAACCACATTGGGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCAT
GAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGG
GTTGTGTACGGAACCTGCCATCACAAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCAC
TAGGAAGCTGCAAACGCGGTCGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGG
ATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCAT
ATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAG
GTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACT
GTCGACATAGAGCTGGTTACAACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCGG
ACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAA
AGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTG
CATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCC
CAGCACAGTGGGATGATCGTTAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATT
CACCAAGAGCCGAAGCCACCCTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCA

GATTTGTATTACTTGACTATGAATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCAC
GCTGGGGCAGACACCGGAACTCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGG
CAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATG
GTGCAAAGGGAAGGCTGTCCTCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATAC
TCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTA
CGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTG
ATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCT
TACATTGTCATAGGAGTCGGGGAGAAGAAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTG
AAGCCACTGTGAGAGGTGCCAAGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCT
CAACTCATTGGGCAAGGGCATCCATCAAATTTTTGGAGCAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACA
AATTCTCATTGGAACGTTGCTGATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGG
GGGAGTGTTGATCTTCTTATCCACAGCTGTCTCTGCTGATGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGAT
GCGGTACAGGGGTGTTCGTCTATAACGACGTTGAAGCCTGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGA
TTGGCAGCAGCAGTCAAGCAAGCCTGGGAAGATGGTATCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTG
GAGATCAGTAGAAGGGGAGCTCAACGCAATCCTGGAAGAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAA
AACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGCCTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAAT
CGTACTTCGTCAGAGCAGCAAAGACAAATAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACAT
AGAGCATGGAACAGCTTTCTTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGA
TTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACT
GGATTGAGAGTGAGAAGAATGACACATGGAGGCTGAAGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAA
AGTCCCACACATTGTGGACAGATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCAT
CACAATACCAGAGAGGGCTACAGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAAT
GCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAG
GGTGATCGAGGAATGGTGCTGCAGGGAGTGCACAATGCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGA
ATGGAGATAAGGCCCAGGAAAGAACCAGAAAGTAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGG
ATCACTTCTCCCTTGGAGTGCTTGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATC
ATAAGCACATCGATGGCAGTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTT
GATGGGTGCCACCTTCGCGGAAATGAACACTGGAGGAGATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGA
CCAGCGTTGCTGGTATCTTTCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTT
TGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGATGGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAG
CGATGGTTGTTCCACGCACTGATAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTG
TGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAA
CTTACCATTTGTCATGGCCCTGGGACTAACCGCTGTGAGGCTGGTCGACCCCATCAACGTGGTGGGACTGCTGTTGCTCAC
AAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTC
GCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGA
GTGTGGACATGTACATTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCT
CGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAG
GTGGTCCTGATGACCATCTGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGAC
TGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTA
CAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGT
GGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATC
TGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCC
CGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCT
GGATTACCCAGCAGGAACTTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTC
GTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGC
CTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAA
ATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGA
AGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAAT
GTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCAC
TTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCAT
GACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAG
AGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAAC
GGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAAACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGT
TCCAGAAAACAAAACATCAAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGAC
CGTGTCATAGATTCCAGGAGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGT

CACACATGCCAGCGCTGCCCAGAGGAGGGGGCGCATAGGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGA
GGTGGGTGCGCAGAGACTGACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGA
TGGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAG
CAAAGGAAGACCTTTGTGGAACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAAT
AACCTACACAGATAGAAGATGGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTG
TGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGA
AGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACA
CATGACAGAGAGATTCCAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAA
GCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTT
TTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATG
TGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAG
CCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTAC
CGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAAC
CATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCA
GCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGT
ATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC
CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGC
GTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACAC
AATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGT
CGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGA
ACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTA
CACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGG
CCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCC
CGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTG
GAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCA
CCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTA
TGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTG
ACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTT
GAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCA
GCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCG
AAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGA
AATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCAT
TGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGG
GCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAA
ACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAG
GAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGA
AAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATT
AGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGT
GGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGA
AACAAGGGGAATTTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGA
AGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGAGAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACA
AAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCAT
TGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGA
CATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGC
AACTCATTCGGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGAC
CAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATT
GATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAAACCCT
CAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCC
ATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGG
AGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCA
ATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAATGG
ATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACCACATGGAAGACAAGACCCCA
GTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGA

CTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTC
AGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTA
TAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACC
CCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGAT
CAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAG

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13 or 72. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13 or 72.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 14
isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 15
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
RDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 16
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXXXXXXXNR
AEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 17
ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMXXXXXGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 18
ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 19
ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 20
isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 21
isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 22
isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVE
FKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA

FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 23
MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 24
LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 25
isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 26
ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 27
AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 28
MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 29
MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 30
KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 31
isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 32
CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 33
Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN

RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 34
isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 35
KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 36
KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 37
isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 38
BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

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SEQ ID NO: 39
MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 40
KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 41
Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 42
KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 43
isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 44
isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 45
PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 46
BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 47
H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 48
PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 49
KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF

EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 50
ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 51
103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 52
isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 53
KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 54
isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 55

isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 56
isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 57
isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 58
isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 59
isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNGTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 60
isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 61
ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 62
KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 63
isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 64
isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCPTQGEAYLD
KQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENR
AKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFK
DAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVE
VQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 65
isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 66
KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 67
isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 68
ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E.
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCPTQGEPSL
DKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 69
MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 70
5'-$(dIdC)_{13}$-3'
dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC

SEQ ID NO: 71
KLK peptide
KLKLLLLLKLK

SEQ ID NO: 72
ZIKV Sequence H/PF/2013 as sequenced
CAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTT
TCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTG
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SEQ ID NO: 73

AHZ13508.1, Zika virus polyprotein from Polynesian outbreak (H/PF/2013)

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9349_Zika_PF_15R
ttaggatccTGTTGTGTTCCTATTGCTGGT

SEQ ID NO: 104
9350_Zika_PF_16F
taactcgaGTGATCAGRGCCCCAGC

SEQ ID NO: 105
9351_Zika_PF_16R
ttaggatccTGCTGCCCAGAAGAGAA

SEQ ID NO: 106
9352_Zika_PF_17F
taactcgaGCACCAACAYGGGTTCTT

SEQ ID NO: 107
9353_Zika_PF_17R
ttaggatcCTCAAGGACGGTGTGGC

SEQ ID NO: 108
9354_Zika_PF_18F
taactcgagCAATGATCTTCATGTTGGG

SEQ ID NO: 109
9355_Zika_PF_18R
ttaggatccTATGGGGGAGGACTGGT

SEQ ID NO: 110
9356_Zika_PF_19F
taactcGAGCCCAGAACCTTGGATC

SEQ ID NO: 111
9357_Zika_PF_19R
ttaggatcCAGACCCCCAAGAAGGC

SEQ ID NO: 112
9358_Zika_PF_20F
taactcgagCCCCTTTGGTCTTGTCT

SEQ ID NO: 113
9359_Zika_PF_20R
ttaggatccAGGAAGGATGTATGCAGATG

SEQ ID NO: 114
9360_Zika_PF_21F
taactcgagACATTTGCGCATATGATTTTG

SEQ ID NO: 115
9361_Zika_PF_21R
ttaggatccAGGAAGGACACACAAGAGT

SEQ ID NO: 116
9362_Zika_PF_22F
taactcgagACAGGCTGCACAGCTTT

SEQ ID NO: 117
9363_Zika_PF_22R
ttaggatccTCTCTCATAGGGCACAGAC

In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NOs: 14-69 or 72. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69 or 72.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. MoI. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Dr., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977 and Altschul et al., J. MoI. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were adapted to ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 9A and B). Briefly, the unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Zika Virus. As shown in Figure 10, non-infectious virus particle aggregates, HCP and other LMW impurities were removed by PS precipitation as shown by removal of aggregate shoulder in SEC-HPLC and no loss of infectious virus titer by PS treatment. Further optimization of the Zika purification protocol is provided below.

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):
- 5% CO_2, 35°C, MEM+2mM L-Glutamine + 10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01

- Virus Production without serum
- 5% CO_2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (accession number KJ776791.1, also SEQ ID NO: 13 herein) from French Polynesia. A sample was obtained from the European Virus Archive (EVAg; Ref-SKU: 001v-EVA1545). Based on this material, a research master seed bank (rMSB) was prepared on Vero cells as the cell substrate and the genomic sequence was checked by sequencing. Because the genomic sequence at the 5´and 3´flanking sequences of Zika virus strain H/PF/2013 was unknown, primers for sequencing were designed in those regions based on other Zika virus strains whereas the internal primers were designed from the published sequence (SEQ ID NOs: 74 to 117, see also Table A). The sequence obtained from the rMSB by use of these primers is provided by SEQ ID NO: 72. There was 100% overlap of the sequence with the published sequence of Zika Virus Strain H/PF/2013 (SEQ ID NO: 13). However, we sequenced additional regions 5´ (an additional 40 bp) and 3' (an additional 160 bp) represented in SEQ ID NO: 72. In a preferred embodiment, the Zika virus of the invention comprises SEQ ID NO: 72. The genomic RNA is somewhat longer than the sequence according to SEQ ID NO: 72 (perhaps an additional 200 bp). Additionally, a Zika virus adapted to a host cell such as e.g. Vero cells may be expected to contain one or more mutations. For these reasons, the Zika virus of the current invention comprises the sequence of SEQ ID NO: 72 or, preferably, a sequence with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 72. Furthermore, because the viral genome is likely to contain even further flanking regions to SEQ ID NO: 72; in one embodiment, the Zika virus of the invention contains the sequence of SEQ ID NO: 72 and optionally further comprises extensions at the 5' and/or 3' ends of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120 or at least 130 nucleotides. In a preferred embodiment, the Zika virus comprises at least the coding sequence for the entire

polyprotein of Zika Virus Strain H/PF/2013 of the invention i.e. the amino acid sequence of SEQ ID NO: 73 or a polyprotein with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 73. Furthermore, the Zika virus comprises at least the coding sequence for the E-protein of Zika Virus Strain H/PF/2013 of the invention SEQ ID NO: 47 or an E-protein thereof with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 47.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in a standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and to reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles were infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200 mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significantly higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with the day 3 harvest, the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest.

Table 3: The calculated titers per plaque assay are summarized in the list below.

	Log 10 PFU/mL
sample day2	7.02
harvest day 3	6.66
harvest day 5	6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 15. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22°C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 11 for SEC-HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 20 for SEC-HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 21). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

Samples of individual 30x concentrated harvests days 2, 3, 5 and 7 were analysed before (Figure 11) and after PS (Figure 12) treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for

determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 4. The virus peak recovery by SEC-HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 4: ZikaV recovery by SEC-HPLC and plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x + PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x + PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles. Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus particles from residual contaminants as demonstrated for ChikV (Fig. 15D). The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 5.

Table 5: Individual layers/volumes for a centrifugation in bottle.

Solution	**Volume (mL)**
PS treated harvest in 10% sucrose (L)	40
15% sucrose (J)	15
35% sucrose (I)	15
50% sucrose (H)	20
Total volume	90

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 14. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled rotor. (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers.) The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no brake/deceleration activated. In case a different centrifuge system with a different rotor is used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle (see Figure 14).

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL)

and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 14. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51 mL in a PETG container. A volume of 1% formaldehyde (10,000 ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200 ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25°C) for a minimum of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criterion for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution was monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to

unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152 µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. Briefly, a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 21.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gently mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 6).

Table 6: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	JEV	Zika
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2 µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565.

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 16. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 17) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6 nm and ~49 nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 18).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Table 7: Active ZikaV pfus were quantified by plaque assay throughout the process.

Sample	Pfu/mL
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$
Harvest day 5 (filtered)	$1.5x10^8$
Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD

Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2 mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly, the Benzonase treated samples did not yield any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell protein was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well was seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5% CO_2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicate. The target viruses (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were diluted to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were

incubated at 35°C with 5% CO_2 for 1 hour. The cell culture medium was aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plates were rocked back and forth 5 times before incubating for 2 hours at 35°C with 5% CO_2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5% CO_2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 19 and 20, respectively). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Another neutralization assay was performed using the microneutralization assay as described by Larocca, et al. (2016, Nature doi:10.1038/nature18952). It was found that the inactivated Zika virus of the current invention had an MN50 (microneutralization) titer of 90 at 1 µg of inactivated purified virus.

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by

administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on Vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV vaccine. The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

The in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-

protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, supra.). Inactivated viruses are among the safest vaccines and especially preferred for deliver to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

A4. The vaccine of any one of A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of A1-A9, further comprising an adjuvant.

A11. The vaccine of A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

A15. The vaccine of any one of A1-A14, further comprising one or more pharmaceutically acceptable excipient.

A16. The vaccine of any one of A1-A15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 μg/mL, especially below 100 ng/mL.

A17. The vaccine of A16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

B1. A kit comprising a Zika virus vaccine of any one of A1-A15.

B2. The kit of B1, further comprising a second vaccine.

B3. The kit of B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of A1-A15 to a subject in need thereof.

C2. The method of C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of A1-A15 for the treatment and/or prevention of a Zika virus infection.

E2. The use of E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment or prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of A1-A15.

F2. The pharmaceutical composition of F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

G1. Use of an optimized sucrose gradient centrifugation for removal of protamine sulphate from purified infectious Zika virus particles.

G2. The use according to G1, wherein said optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three further layers of sucrose solutions with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G3. A process of purification of infectious Zika virus particles, comprising the steps of:

a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

G4. The process of G3, wherein said optimized sucrose density gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G5. The process of any one of G3 to G4, additionally comprising a further purification step of: (d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from

entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

G6. The process of any of G3 to G5, wherein the residual host cell DNA content of the Zika virus preparation (c) is less than 10 ng/mL and the residual host cell protein content of the final virus preparation (c) is less than 100 ng/mL.

G7. The process of any of G3 to G6, wherein said crude harvest (a) comprising Zika virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

G8. The process of G7, wherein the one or more pre-purification step(s) comprises

a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

b) digestion of host cell genomic DNA by enzymatic treatment; and/or

c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

G9. The process of any one of G3 to G8, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

G10. The process of any one of G3 to G9, wherein the enrichment of infectious Zika virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

G11. The process of any one of G7 to G10, wherein the one or more pre-purification step(s) prior to step (b) of any of G8 to G11 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

G12. The process of any one of G3 to G11, wherein the residual impurity of the Zika virus preparation (c) is less than 10%.

G13. The process of any one of G3 to G12, wherein the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a

PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

G14. The process of G13, wherein said cell line is a Vero cell line.

G15. The process of any one of G3 to G14, wherein said infectious Zika virus particle is an infectious virus particle that is a live virus, a live attenuated virus, a chimeric virus, a modified live virus, or a recombinant live virus.

G16. The process of any one of G3 to G15, wherein said Zika virus is preferably a strain of the Asian lineage.

G17. The process of any one of G3 to G16, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

G18. Use of the process according to any one of G3 to G17 for manufacturing a composition for immunization against a virus infection.

G19. The use according to G18, wherein said virus infection is an infection caused by a Zika virus.

Q1. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

Q2. The process of Q2, wherein the virus particles are from Zika virus.

Q3. The process of Q1 or Q2, additionally comprising the step of:

(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

Q4. The process of any of Q1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

Q5. The process of any of Q1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

Q6. The process of Q5, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

Q7. The process of any one of Q1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

Q8. The process of any one of Q1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

Q9. The process of any one of Q5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of Q5 to 8 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

Q10. The process of any one of Q1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

Q11. The process of any one of Q1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

Q12. The process of Q11, wherein said cell line is a Vero cell line.

Q13. The process of any one of Q1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

Q14. The process of any one of Q1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

Q15. The process of any one of Q1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

Q16. Use of the process according to any one of Q1 to 15 for manufacturing a composition for immunization against a virus infection.

Q17. The use according to Q16, wherein the composition for immunization against a virus infection is an infection caused by Zika virus.

Q18. A composition comprising the virus particles obtainable or obtained by the process of any one of Q1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

Q19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

Q20. The Zika virus vaccine of Q19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

Q21. The vaccine of Q19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

Q22. The vaccine of any one of Q19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69 or 72, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 or 72 and able to pack a virulent Zika virus.

Q23. The vaccine of any one of Q19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

Q24. The vaccine of Q23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

Q25. The vaccine of Q24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

Q26. The vaccine of any one of Q19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

Q27. The vaccine of Q26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

Q28. The vaccine of Q27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

Q29. The vaccine of Q28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

Q30. The vaccine of any one of Q27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

Q31. The vaccine of any one of Q19 to 30, further comprising an adjuvant.

Q32. The vaccine of Q31, wherein the adjuvant is an aluminum salt adjuvant.

Q33. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

Q34. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

Q35. The vaccine of any one of Q19 to 34, further comprising one or more pharmaceutically acceptable excipient.

R1. Use of protamine, preferably a protamine salt, to separate infectious and non-infectious Zika virus particles, host cell proteins and/or undefined low molecular weight materials.

R2. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b), wherein the enrichment of infectious virus particles in the virus preparation (b) relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R3. The use of R1 or the process of R2, wherein the virus particles are from Zika virus.

R4. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by one or more size exclusion methods such as (i) a sucrose density gradient centrifugation, (ii) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles, and/or (iii) size exclusion chromatography to obtain a virus preparation (c) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (c) is less than 100 ng/mL and the residual host cell protein and the residual aggregates of infectious virus particles of the final virus preparation (c) is less than 1 μg/mL.

R5. The process of R4, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

R6. The process of any of R2 to 5, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

R7. The process of R6, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

R8. The process of any one of R2 to 7, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.

R9. The process of any one of R2 to 8, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R10. The process of any one of R6 to 9, wherein the one or more pre-purification step(s) prior to step (b) of any of R6 to 9 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

R11. The process of any one of R2 to 10, wherein the residual impurity of the virus preparation (c) is less than 10%.

R12. The process of any one of R2 to 11, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

R13. The process of R12, wherein said cell line is a Vero cell line.

R14. The process of any one of R2 to 13, wherein the Zika virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

R15. The process of any one of R2 to 14, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

R16. The process of any one of R2 to 15, wherein said process resulting in final virus preparation (c) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

R17. Use of the process according to any one of R1 to 16 for manufacturing a composition for immunization against a virus infection.

R18. The use according to R17, wherein the composition for immunization against a virus infection is an infection caused by a Zika virus.

R19. A composition comprising the virus particles obtainable or obtained by the process of any one of R2 to 16 for treating and/or preventing an infection.

Electronic Patent Application Fee Transmittal

Application Number:	16063007
Filing Date:	15-Jun-2018
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Filer:	Chelsea E. Witte-Garcia/Katelyn Farmer
Attorney Docket Number:	I0422.70125US00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

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Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
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			Total in USD ($)	**1000**

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Application Number:	16063007
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First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
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Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	I042270125US00-ISSFEE-CEW.pdf	36803 173d36dcd32b2863a31f4d2146a11989e146522e	no	1
Warnings:					
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3		I042270125US00-312AMN-CEW.pdf	29722 abe2bc603d07212b5eb9757f8b7042d9ccf8d7b4	yes	3

Multipart Description/PDF files in .zip description

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Applicant Arguments/Remarks Made in an Amendment	3	3

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5	Specification	I042270125US00-SUBSPEC-CEW.pdf	684063 783cf4e4a8fc280ebe1c625ad8b384e9c9df41eb	no	99

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	1648
Examiner Name	B. A. Chestnut
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

-Part B – Fee(s) Transmittal
-Amendment After Allowance
-Substitute Specification (Clean and Marked-Up Copies)

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Chelsea E. Witte-Garcia/		
Printed name	Chelsea E. Witte-Garcia, Ph.D.		
Date	February 13, 2020	Reg. No.	75,713

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: February 13, 2020 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

PART B–FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is ~~being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or~~ being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

Katelyn R. Farmer	(Typed or printed name)
/Katelyn R. Farmer/	(Signature)
February 13, 2020	(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
16/063,007	06/15/2018	Jana Barbero Calzado	I0422.70125US00	4503

TITLE OF INVENTION: ZIKA VIRUS VACCINE

APPLN: TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	04/30/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
B. A. Chestnut	1648	424-218100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363)

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. Wolf, Greenfield & Sacks, P.C.
2.
3.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Valneva Austria GmbH

(B) RESIDENCE: (CITY and STATE or COUNTRY): Vienna, Austria

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment (*Please first reapply any previously paid fee shown above*):

☒ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge ~~the required fee(s),~~ any deficiency, or credit any overpayment to Deposit Account No. 23/2825

5. Change of Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29. NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27. NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status. NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Chelsea E. Witte-Garcia/ Date February 13, 2020

Typed or printed name Chelsea E. Witte-Garcia, Ph.D. Registration No. 75,713

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15 2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 02 20/2020
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER
CHESTNUT, BARRY A

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
02/20/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 16063007
Applicant : Calzado
Filing Date : 06/15/2018
Date Mailed : 02/20/2020

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Kam Sin/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 16063007 **Drawings filed: 06/15/2018**

DRAWINGS AND BRIEF DESCRIPTION OF DRAWINGS IN NON-COMPLIANCE WITH 37 CFR 1.84(u)(1)

37 CFR 1.84(u)(1) states, "The different views must be numbered in consecutive Arabic numerals, starting with 1 … Partial views intended to form one complete view, on one or several sheets, must be identified by the same number followed by a capital letter. View numbers must be preceded by the abbreviation 'FIG.' "

Other methods of identification are improper, including Roman numerals (FIG. I, FIG, II, etc.) and alphabetic characters (FIG. A, FIG. B, etc.). Abbreviations and words other than "FIG." ("Illustration, 1" "Diagram 2," "Photo 3," etc.) are improper.

☐ Applicant must submit drawings that number the views using consecutive Arabic numerals.

☐ Applicant must amend the brief description of the drawings on Page(s) of the specification to show consecutive Arabic numerals.

NOTE: Applicant is advised that the detailed description may voluntarily be amended to identify the drawings by Arabic numerals.

☒ The drawing is continued onto a second page (or more) without proper labeling under 37 CFR 1.84(u)(1). FIG(s) 7

☐ Applicant must submit drawings on which the view numbers are preceded by the abbreviation "FIG."

☐ Applicant must amend the brief description of the drawings on Page(s) of the specification to show view numbers preceded by the abbreviation "FIG."

NOTE: Applicant is advised that the detailed description may voluntarily be amended to show view numbers preceded by the abbreviation "FIG."

PATENT ASSIGNMENT COVER SHEET

Electronic Version v1.1
Stylesheet Version v1.2

EPAS ID: PAT5997036

SUBMISSION TYPE:	NEW ASSIGNMENT
NATURE OF CONVEYANCE:	SECURITY INTEREST

CONVEYING PARTY DATA

Name	Execution Date
VALNEVA SE	03/04/2020
VALNEVA USA, INC.	03/04/2020
VALNEVA AUSTRIA GMBH	03/04/2020

RECEIVING PARTY DATA

Name:	WILMINGTON TRUST, NATIONAL ASSOCIATION
Street Address:	50 SOUTH SIXTH STREET, SUITE 1290
City:	MINNEAPOLIS
State/Country:	MINNESOTA
Postal Code:	55402

PROPERTY NUMBERS Total: 41

Property Type	Number
Patent Number:	9598472
Patent Number:	10357557
Application Number:	16295031
Patent Number:	7148191
Patent Number:	8361476
Patent Number:	8900564
Patent Number:	8568742
Patent Number:	8945591
Patent Number:	9492537
Patent Number:	7244438
Patent Number:	7704514
Patent Number:	8784837
Patent Number:	9884115
Patent Number:	9913898
Application Number:	15469689
Application Number:	16734866
Patent Number:	9895437
Patent Number:	8986704

Property Type	Number
Patent Number:	9926343
Patent Number:	10544194
Application Number:	15906618
Application Number:	16702885
Patent Number:	9975927
Application Number:	15944835
Application Number:	16063007
Application Number:	16497178
Application Number:	15781825
Application Number:	16060054
Patent Number:	9382513
Application Number:	13206056
Patent Number:	8148132
Patent Number:	9040296
Patent Number:	9701945
Patent Number:	8962311
Patent Number:	9260694
Patent Number:	9822345
Application Number:	15785530
Application Number:	15561094
Patent Number:	10537630
Application Number:	16702764
Application Number:	15781959

CORRESPONDENCE DATA

Fax Number:

Correspondence will be sent to the e-mail address first; if that is unsuccessful, it will be sent using a fax number, if provided; if that is unsuccessful, it will be sent via US Mail.

Email: akwon@cov.com
Correspondent Name: COVINGTON & BURLING LLP
Address Line 1: ONE CITY CENTER, 850 TENTH ST NW
Address Line 4: WASHINGTON, D.C. 20001

ATTORNEY DOCKET NUMBER:	034550.00068
NAME OF SUBMITTER:	ASHLEY M. KWON
SIGNATURE:	/ASHLEY M. KWON/
DATE SIGNED:	03/04/2020
	This document serves as an Oath/Declaration (37 CFR 1.63).

Total Attachments: 15
source=Patent Security Agreement#page1.tif

source=Patent Security Agreement#page2.tif
source=Patent Security Agreement#page3.tif
source=Patent Security Agreement#page4.tif
source=Patent Security Agreement#page5.tif
source=Patent Security Agreement#page6.tif
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source=Patent Security Agreement#page14.tif
source=Patent Security Agreement#page15.tif

PATENT SECURITY AGREEMENT

This PATENT SECURITY AGREEMENT, dated as of March 4, 2020 (this "Agreement"), is made by the Grantors listed on the signature pages hereto (each a "Grantor" and collectively, the "Grantors"), in favor of Wilmington Trust, National Association, as Administrative Agent (in such capacity, the "Administrative Agent") for the Secured Parties.

W I T N E S S E T H :

WHEREAS, pursuant to the Credit Agreement, dated as of February 3, 2020 (as amended, restated, supplemented or otherwise modified from time to time, the "Credit Agreement"), by and among the Borrower, Holdings, the Lenders (as defined therein), and the Administrative Agent, the Lenders have extended Commitments to make Loans to the Borrower;

WHEREAS, in connection with the Credit Agreement, each Grantor and its Affiliates have executed and delivered a Pledge and Security Agreement in favor of the Administrative Agent, for the benefit of the Secured Parties, dated as of March 4, 2020 (as amended, supplemented or otherwise modified from time to time, the "Security Agreement");

WHEREAS, pursuant to the Credit Agreement and pursuant to clause (e) of Section 4.5 of the Security Agreement, each Grantor is required to execute and deliver this Agreement and to grant to the Administrative Agent, for the benefit of the Secured Parties, a continuing security interest in all of the Patent Collateral (as defined below) to secure all of the Obligations; and

WHEREAS, each Grantor has duly authorized the execution, delivery and performance of this Agreement;

NOW, THEREFORE, for good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged, each Grantor agrees, for the benefit of the Administrative Agent, as follows:

Section 1 Definitions. Unless otherwise defined herein or the context otherwise requires, terms used in this Agreement, including its preamble and recitals, have the meanings provided (or incorporated by reference) in the Security Agreement.

Section 2 Grant of Security Interest. Each Grantor hereby grants to the Administrative Agent, for the benefit of the Secured Parties, a continuing security interest in all of such Grantor's right, title and interest in and to the following property, whether now or hereafter existing or acquired by such Grantor (the "Patent Collateral"):

(a) all of its letters patent and applications for letters patent throughout the world, including each patent and patent application referred to in Item A of Schedule I attached hereto;

(b) all reissues, divisions, continuations, continuations-in-part, extensions, renewals and reexaminations of any of the items described in clause (a);

(c) all patent licenses and other agreements providing such Grantor with the right to use any of items of the type referred to in clauses (a) and (b) above, including each exclusive patent license referred to in Item B of Schedule I attached; and

(d) all Proceeds of, and rights associated with, the foregoing (including royalties income, payments, claims, damages and Proceeds of infringement suits) and the right to sue third

parties for past, present or future infringements of any patent or patent application and for breach or enforcement of any patent license.

Section 3 Security Agreement. This Agreement has been executed and delivered by each Grantor for the purpose of registering the security interest of the Administrative Agent in the Patent Collateral with the United States Patent and Trademark Office. The security interest granted hereby has been granted in furtherance of, and not in limitation of, the security interest granted to the Administrative Agent for the benefit of the Secured Parties under the Security Agreement. The Security Agreement (and all rights and remedies of the Secured Parties thereunder) shall remain in full force and effect in accordance with its terms.

Section 4 Release of Liens. Upon (a) the Disposition of Patent Collateral in accordance with the Credit Agreement or (b) the occurrence of the Termination Date, the security interest granted herein shall automatically terminate with respect to (i) such Patent Collateral (in the case of clause (a)) or (ii) all Patent Collateral (in the case of clause (b)). Upon any such Disposition or termination, the Administrative Agent will, at the Grantors' sole expense, deliver to the Grantor or Grantors, as applicable, without any representations, warranties or recourse of any kind whatsoever, all Patent Collateral held by the Administrative Agent hereunder, and execute and deliver to the Grantor or Grantors, as applicable, such documents as the Grantor or Grantors shall reasonably request to evidence such termination.

Section 5 Acknowledgment. Each Grantor does hereby further acknowledge and affirm that the rights and remedies of the Secured Parties with respect to the security interest in the Patent Collateral granted hereby are more fully set forth in the Security Agreement, the terms and provisions of which (including the remedies provided for therein) are incorporated by reference herein as if fully set forth herein.

Section 6 Loan Document. This Agreement is a Loan Document executed pursuant to the Credit Agreement and shall (unless otherwise expressly indicated herein) be construed, administered and applied in accordance with the terms and provisions thereof, including Article X thereof.

Section 7 Effectiveness. This Agreement shall become effective when a counterpart hereof executed by each Grantor, shall have been received by the Administrative Agent. Delivery of an executed counterpart of a signature page to this Agreement by email (in "pdf," "tiff" or similar format) or telecopy shall be effective as delivery of a manually executed counterpart of this Agreement.

[*Signature Page Follows*]

IN WITNESS WHEREOF, each Grantor hereto has caused this Agreement to be duly executed and delivered by its Authorized Officer(s) as of the date first above written.

VALNEVA SE

By: _____
Name: Frederic Jacobet
Title: General Counsel

[*Signature Page to Patent Security Agreement*]

VALNEVA USA, INC.

By: _____

Name: Thomas Lingelbach
Title: CEO

[Signature Page to Patent Security Agreement]

VALNEVA AUSTRIA GMBH

By: _____

Name: David Lawrence
Title: Managing Director

By: _____

Name: Frédéric Jacotot
Title: Managing Director

[Signature Page to Patent Security Agreement]

WILMINGTON TRUST, NATIONAL ASSOCIATION,
as Administrative Agent

By: _____
Name: Jessica Jankiewicz
Title: Banking Officer

A. Patents

i) Valneva USA

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP091/US	United States of America	US14/342,565 05-Sep-2011	US-2015-0056238-A1 26-Feb-2015	US9598472 21-Mar-2017	Granted	
ICP091/US/1	United States of America	US15/421,808 05-Sep-2011	US-2017-0239340-A1 24-Aug-2017	US 10,357,557 23-Jul-2019	Granted	
ICP091/US/2	United States of America	16/295,031 07-Mar-2019	US-2019-0290747-A1 26-Sep-2019		Pending	Published

ii) Valneva Austria GmbH

Short Title: TransVax + Poly IC

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP010/US	United States of America	US10/297,497 07-Jun-2001	US-2003-0162738-A1 28-Aug-2003	US7148191 12-Dec-2006	Granted	

Short Title: Synthetic KLK Peptides

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP013/US	United States of America	US10/399,442	US-2005-0063978-A1	US8361476	Granted	

		18-Oct-2001	24-Mar-2005	29-Jan-2013		
ICP013/US/1	United States of America	US12/124,785	US-2009-0123486-A1	US8900564	Granted	
		18-Oct-2001	14-May-2009	02-Dec-2014		

Short Title: I-ODNs

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP015/US	United States of America	US10/297,555	US-2003-0171321-A1	US8568742	Granted	
		07-Jun-2001	11-Sep-2003	29-Oct-2013		
ICP015/US/1	United States of America	US13/786,815	US-2013-0183339-A1	US8945591	Granted	
		07-Jun-2001	18-Jul-2013	03-Feb-2015		
ICP015/US/2	United States of America	US14/585,740 06-Mar-2013	US-2015- 07-May-2015	US9492537 15-Nov-2016	Granted	

Short Title: Depot

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP020/US	United States of America	US10/250,953	US-2004-0071719-A1	US7244438	Granted	
		07-Jan-2002	15-Apr-2004	17-Jul-2007		

Short Title: IC31 + viral vaccine

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP039/US	United States of America	US10/550,754	US-2006-0263386-A1	US7704514	Granted	
		22-Mar-2004	23-Nov-2006	27-Apr-2010		
ICP039/US/1	United States of America	US12/759,318	US-2010-0297170-A1	US8784837	Granted	
		22-Mar-2004	25-Nov-2010	22-Jul-2014		

Short Title: C. diff. G5 construct

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP091/US	United States of America	US14/342,565 05-Sep-2011	US-2015-0056238-A1 26-Feb-2015	US9598472 21-Mar-2017	Granted	
ICP091/US/1	United States of America	US15/421,808 05-Sep-2011	US-2017-0239340-A1 24-Aug-2017	US 10,357,557 23-Jul-2019	Granted	
ICP091/US/2	United States of America	16/295,031 07-Mar-2019	US-2019-0290747-A1 26-Sep-2019		Pending	Published

Short Title: Improved alum

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP104/US/2	United States of America	US14/873,344 18-Apr-2012	US20160114033A1 28-Apr-2016	US9884115 06-Feb-2018	Granted	
ICP104/US/3	United States of America	US15/223,027 29-Jul-2016	US2016-0324959 10-Nov-2016	US9913898 13-Mar-2018	Granted	
ICP104/US/4	United States of America	US15/469,689 18-Apr-2012	US-2017-0296656-A1 19-Oct-2017	US10,568,959 25-Feb-2020	Granted	
ICP104/US/5	United States of America	US16/734,866 06-Jan-2020			Pending	
ICP104/US-PR1	United States of America	US13/449,596 18-Apr-2012	US2013-0280295A1 24-Oct-2013	US9895437 20-Feb-2018	Granted	

Short Title: OspA vaccine against Borrelia

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
ICP107/US	United States of America	US13/802,991 14-Mar-2013	US20140010835A1 09-Jan-2014	US8,986,704 24-Mar-2015	Granted	
ICP107/US/3	United States of America	US15/267,372	US-2017-0107263-A1	US9,926,343	Granted	

		14-Mar-2013	20-Apr-2017	27-Mar-2018		
ICP107/US/4	United States of America	US15/272,581	US-2017-0101446-A1	US10,544,194	Granted	
		08-Jul-2013	13-Apr-2017	28-Jan-2020		
ICP107/US/5	United States of America	US15/906,618	US-2018-0327460-A1		Pending	Published
		14-Mar-2013	15-Nov-2018			
ICP107/US/6	United States of America	US16/702,885			Pending	
		08-Jul-2013				

Short Title: Chimeric short stabilized OspA for improvement of expression and solubility of OspA serotype 3 containing monomer and heterodimer

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT003/US	United States of America	US15/110,151	US-2016-0333056-A1	US9,975,927	Granted	
		09-Jan-2015	17-Nov-2016	22-May-2018		
PAT003/US/1	United States of America	US15/944,835	US-2018-0362593-A1		Pending	Published
		09-Jan-2015	20-Dec-2018			

Short Title: Inactivated Zika virus vaccine

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT018/US	United States of America	US16/063,007 23-Dec-2016	US2018-0371027 27-Dec-2018		Pending	Published

Short Title: Multivalent OspA single antigen for vaccination against Lyme borreliosis

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT024/US	United States of America	US16/497,178 13-Apr-2018	WO2018/189372 18-Oct-2018		Pending	Nat. Phase

Short Title: Zika-specific protamine sulfate

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT025/US	United States of America	US15/781,825 23-Dec-2016	US-2018-0362936-A1 20-Dec-2018		Pending	Published

Short Title: Zika-specific sucrose gradient

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT026/US	United States of America	US16/060,054 23-Dec-2016	US-2019-0008945-A1 10-Jan-2019		Pending	Published

iii) Valneva SE

Short Title: Lignées de cellules aviaires utiles pour la production de substances d'intérêt

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
B003/US/3	United States of America	US12/717,096 07-Mar-2003	US20100221825 02-Sep-2010	US9382513 05-Jul-2016	Granted	
B003/US/4 (EXAM)	United States of America	US13/206,056 07-Mar-2003	US20110294209 01-Dec-2011		Pending	Appealed

Short Title: Process of manufacturing viral vaccines in suspension in avian embryonic derived stem cells line

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
B014/US	United States of America	US11/918,206 11-Apr-2006	US-2009-0081251 26-Mar-2009	US8148132 03-Apr-2012	Granted	

B014/US/1	United States of America	US13/423,195 11-Apr-2006	US20120238001 20-Sep-2012	US9040296 26-May-2015	Granted	
B014/US/2	United States of America	US14/718,393 21-May-2015	US2015/0275186A1 01-Oct-2015	US9701945 11-Jul-2017	Granted	

Short Title: Method of Producing Transgenic Chicken using ES cells

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
B016/US	United States of America	US12/376,660 09-Aug-2007	US20100235937 16-Sep-2010	US8962311 24-Feb-2015	Granted	

Short Title: Duck embryonic derived stem cell lines for the production of viral vaccines

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
B020/US/1	United States of America	US14/069,423 01-Nov-2013	US2014-0154741 05-Jun-2014	US9260694 16-Feb-2016	Granted	

Short Title: Duck embryonic derived stem cell lines for the production of viral vaccines

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
B020/US/2	United States of America	US15/013,079 02-Feb-2016	US20160257940 08-Sep-2016	US9822345 21-Nov-2017	Granted	
B020/US/3	United States of America	US15/785,530 02-Feb-2016	US 20180135026 17-May-2018		Pending	Published

Short Title: Aseptic Purification Process for Viruses

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT012/US	United States of America	US15/561,094 04-Apr-2016	US20180119110 03-May-2018		Pending	Published

Short Title: Alphavirus Purification

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT015/US	United States of America	US16/062,245 23-Dec-2016	US-2018-0369359-A1 27-Dec-2018	US10,537,630 21-Jan-2020	Granted	
PAT015/US/1	United States of America	US16/702,764 23-Dec-2016			Pending	

Short Title: PS use in virus purification

Docket ID	*Country*	*Application No* *Application Date*	*Publication No* *Publication Date*	*Grant No* *Grant Dt*	*Status*	*Sub-Status*
PAT022/US	United States of America	US15/781,959 23-Dec-2016	US-2018-0362937-A1 20-Dec-2018		Pending	Published

B. Patent Licenses

In-licensed patents related to IXIARO® Valneva Austria

Docket ID	Short Title	Application No	Application Date	Grant No	Grant Date	SPC holder
CJC001/EP/BE-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	2009C/037	22.9.2009			Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/DE-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	12 2009 000 037.0	14.07.2009	12 2009 000 037.0	16.06.2011	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/DK-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	CA 2009 00020	17.07.2009	CA 2009 00020	11.06.2012	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/ES-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	C 200900035	04.09.2009	C 200900035	18.04.2011	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/FR-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	FR09C0037	14.08.2009	FR09C0037	14.08.2009	Cheil Jedang Corporation, Walter Reed Army Institute of Research

Docket ID	Short Title	Application No	Application Date	Grant No	Grant Date	SPC holder
CJC001/EP/GB-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	SPC/GB09/033	30.07.2009	SPC/GB09/033	15.06.2011	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/IT-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	UB2009CCP1.118	17.09.2009	UB2009CCP1.118	24.11.2009	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/NL-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	300391	10.06.2009	300391	07.09.2009	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/US-EXT	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	FDA-2009-E-0416	28-May-2009	FDA-2009-E-0416	31-Jul-2012	Cheil Jedang Corporation, United States Army
CJC001/AU-EXT	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	748730	26.05.2009	748730	19.06.2009	Cheil Jedang Corporation, Walter Reed Army Institute of Research
CJC001/EP/1/CH-SPC	An attenuated Japanese Encephalitis virus adapted to vero cell and a Japanese Encephalitis vaccine	C01604685/01	20.08.2012			Cheil Jedang Corporation, Walter Reed Army Institute of Research

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Form Revision Date: March 1, 2019

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	B. A. Chestnut
Art Unit:	1648

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Dated: March 10, 2020 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

AMENDMENT IN RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Notice to File Corrected Application Papers dated February 20, 2020, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Drawings begin on page 3 of this paper

Remarks begin on page 4 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace the previously-filed specification with the concurrently filed Substitute Specification (clean version). The Substitute Specification contains no new matter.

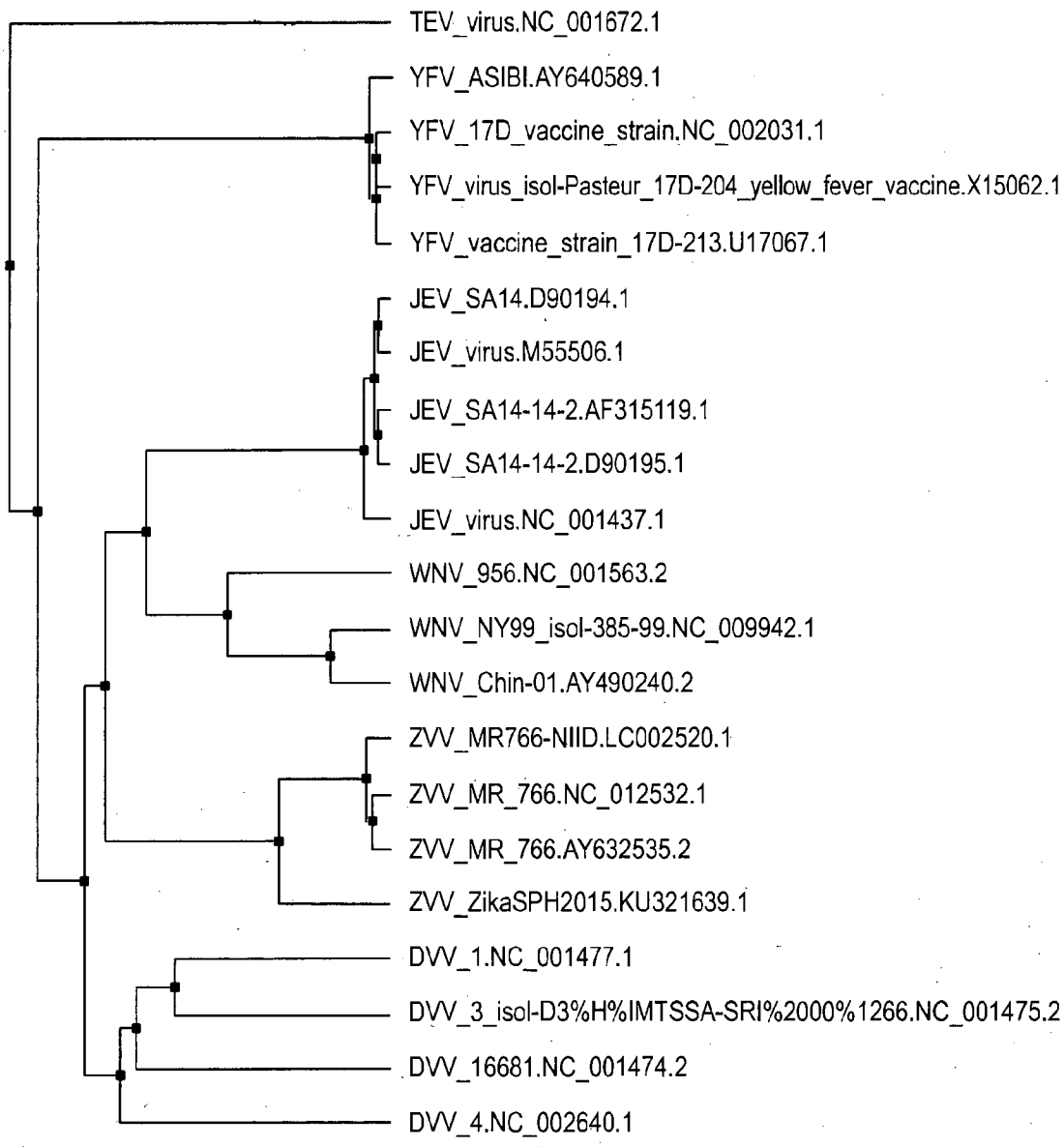

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YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
DVV_1.NC_001477.1
DVV_3_isol-D3%H%IMTSSA-SRI%2000%1266.NC_001475.2
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Figure 1

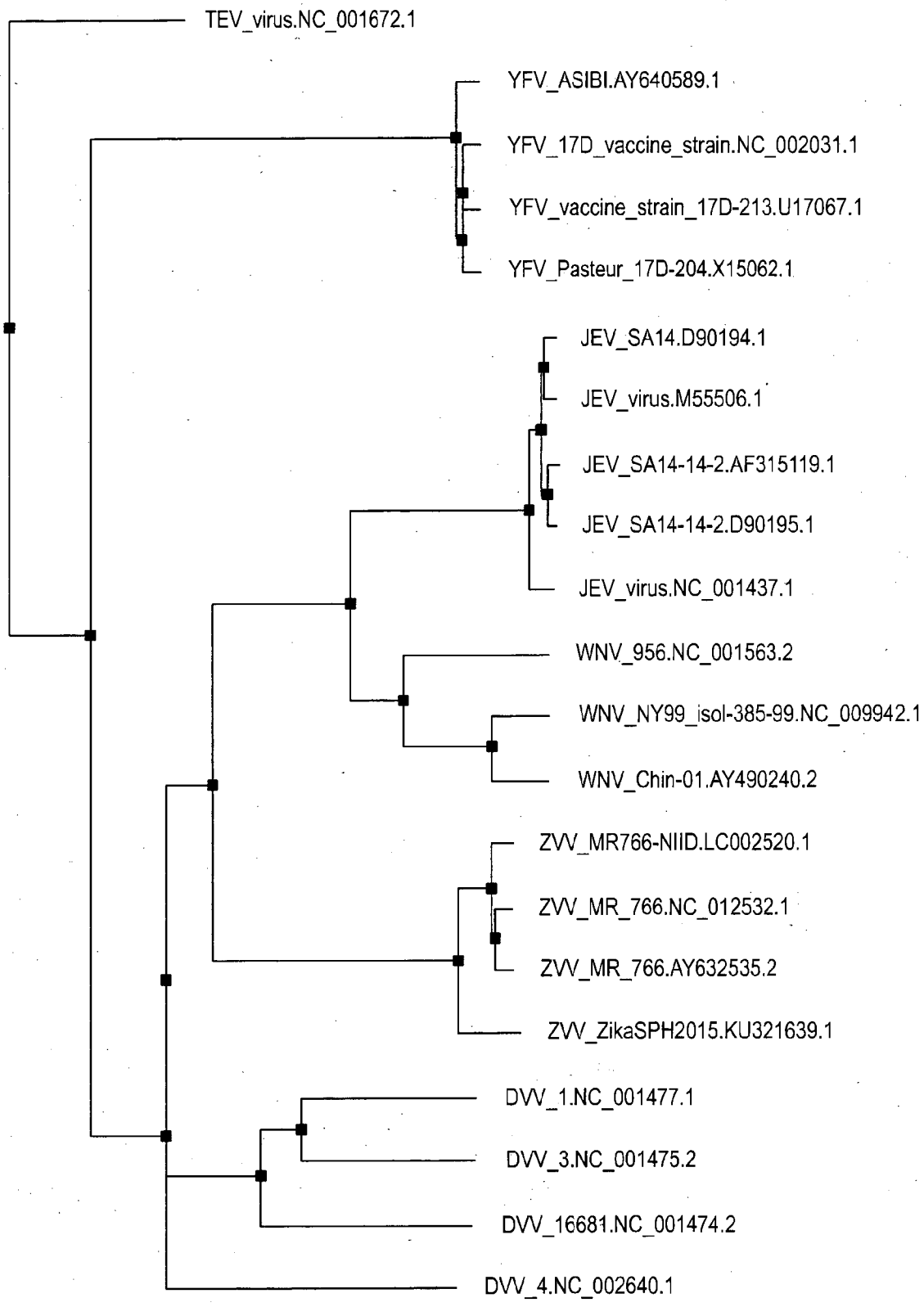

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YFV_Pasteur_17D-204.X15062.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
ZVV_MR766-NIID.LC002520.1
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Figure 2

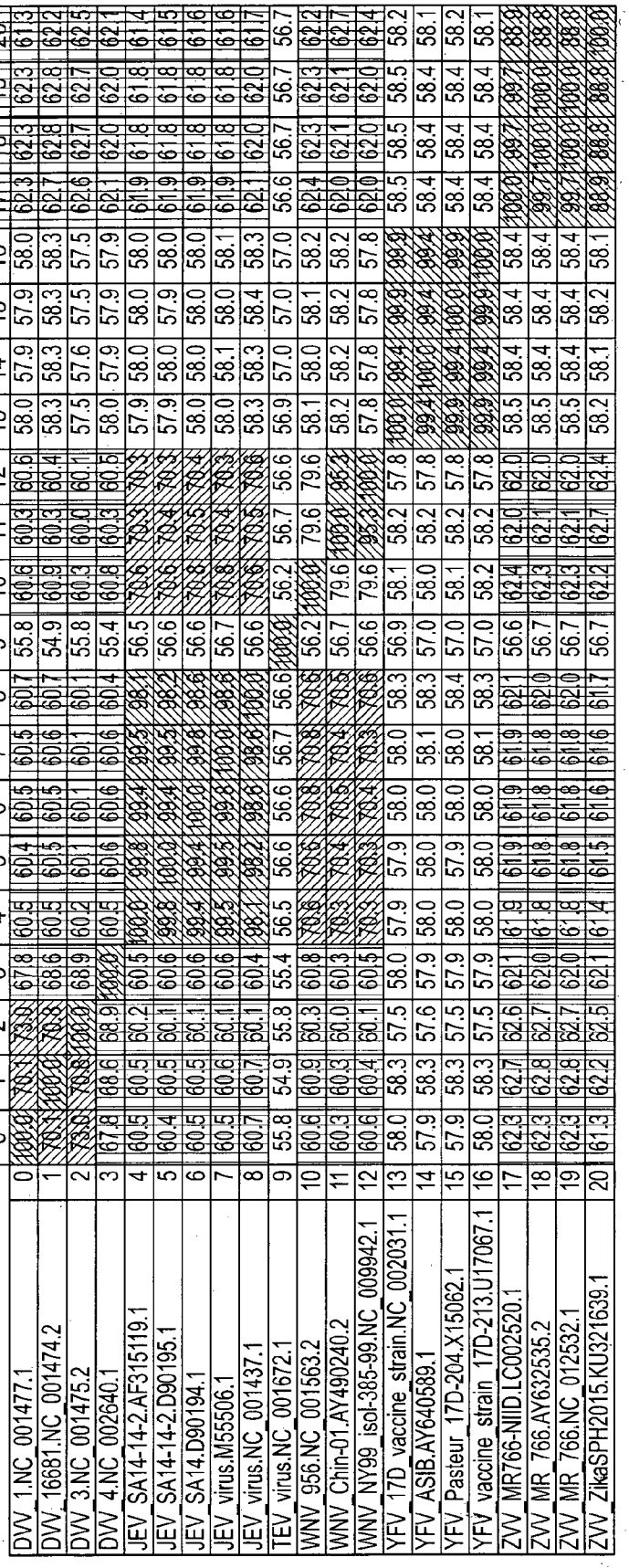

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DVV_3.NC_001475.2 2
DVV_4.NC_002640.1 3
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JEV_SA14-14-2.D90195.1 5
JEV_SA14.D90194.1 6
JEV_virus.M55506.1 7
JEV_virus.NC_001437.1 8
TEV_virus.NC_001672.1 9
WNV_956.NC_001563.2 10
WNV_Chin-01.AY490240.2 11
WNV_NY99_isol-385-99.NC_009942.1 12
YFV_17D_vaccine_strain.NC_002031.1 13
YFV_ASIB.AY640589.1 14
YFV_Pasteur_17D-204.X15062.1 15
YFV_vaccine_strain_17D-213.U17067.1 16
ZVV_MR766-NIID.LC002520.1 17
ZVV_MR_766.AY632535.2 18
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% Identity and higher
% Identity and higher
% Identity and higher

Figure 3

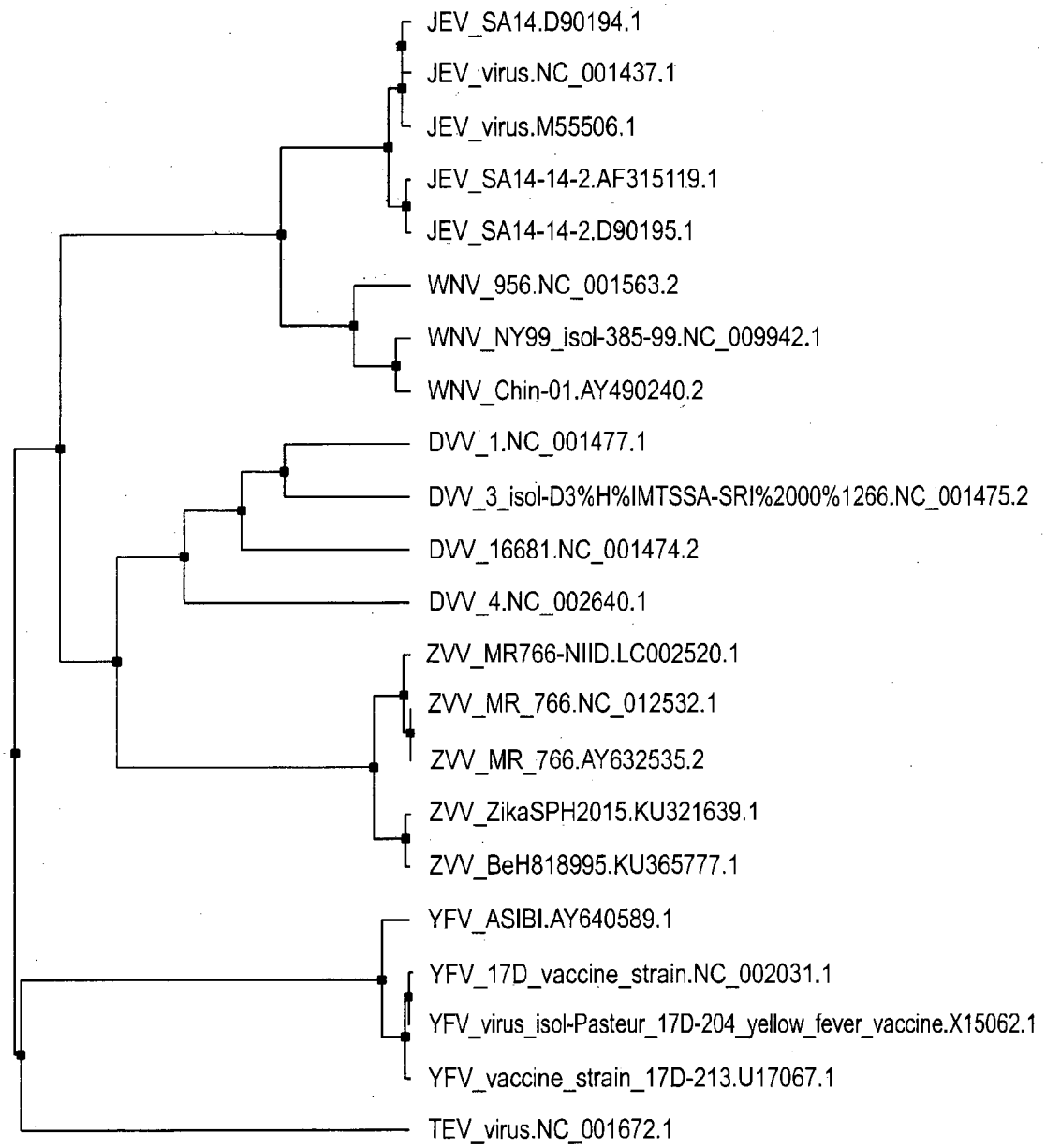

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JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
DVV_1.NC_001477.1
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DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
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ZVV_BeH818995.KU365777.1
YFV_ASIBI.AY640589.1
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YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1

Figure 4

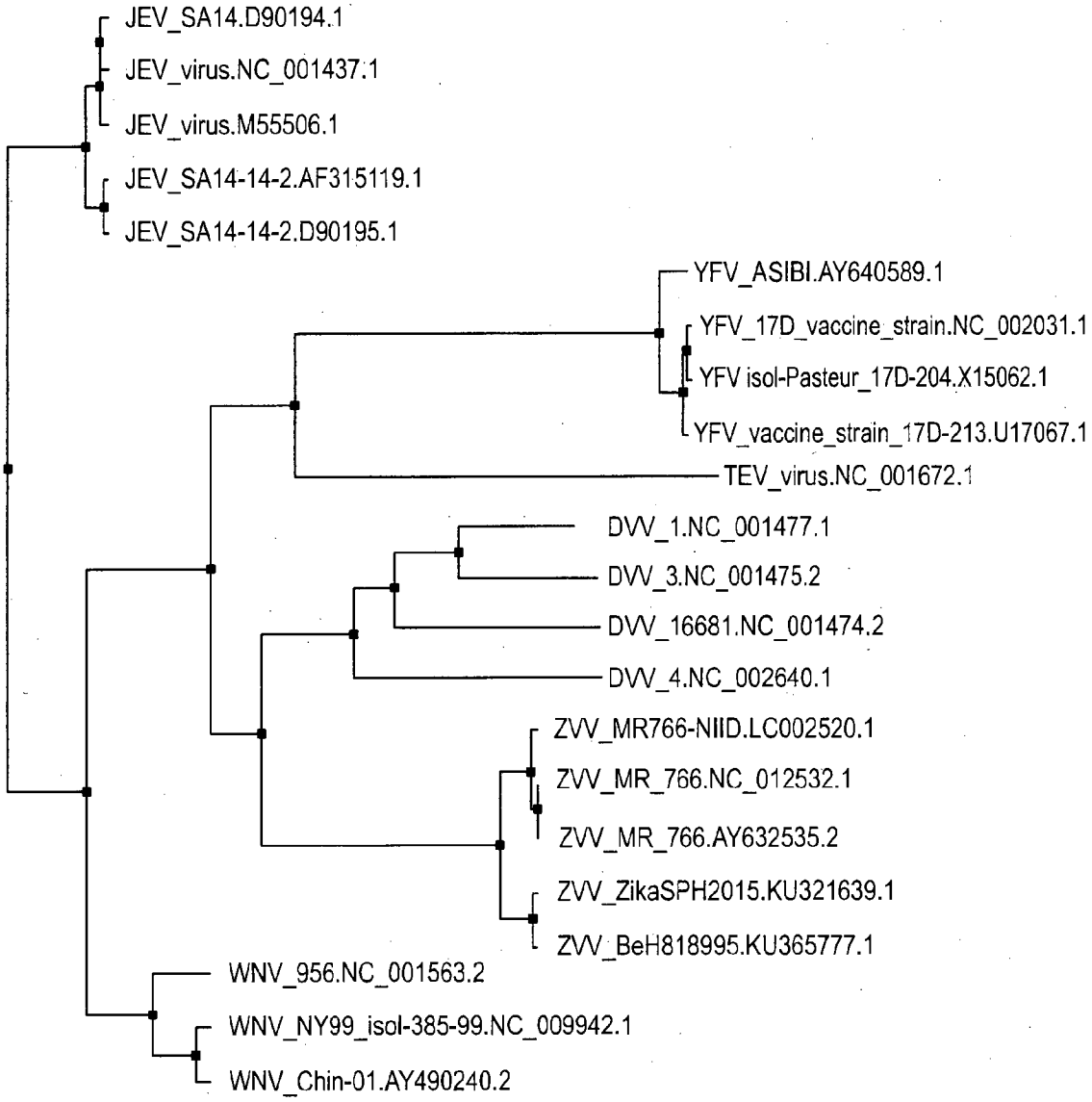

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YFV_17D_vaccine_strain.NC_002031.1
YFV isol-Pasteur_17D-204.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1
DVV_1.NC_001477.1
DVV_3.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
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ZVV_BeH818995.KU365777.1
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WNV_Chin-01.AY490240.2

Figure 5

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DVV_1.NC_001477.1	0	100.0	68.7	77.4	63.8	49.7	49.7	50.1	49.9	50.5	38.3	51.5	50.5	50.9	43.0	43.8	43.0	43.2	57.3	57.9	58.4	58.4	57.1
DVV_16681.NC_001474.2	1	68.7	100.0	68.7	64.0	46.7	46.7	47.1	46.9	47.5	38.9	48.4	47.6	47.6	43.6	44.4	43.6	43.6	53.6	53.8	54.2	54.2	53.8
DVV_3.NC_001475.2	2	77.4	68.7	100.0	62.8	48.0	48.0	48.2	48.2	48.6	38.2	46.8	46.7	46.3	41.6	42.2	41.6	41.7	57.3	57.7	58.2	58.2	57.1
DVV_4.NC_002640.1	3	63.8	64.0	62.8	100.0	47.5	47.5	47.7	47.5	48.1	39.7	49.6	49.6	49.8	40.0	40.6	40.0	40.0	55.8	56.4	56.4	56.4	55.8
JEV_SA14-14-2.AF315119.1	4	49.7	46.7	48.0	47.5	100.0	99.8	98.0	98.6	97.8	39.6	76.1	76.5	76.7	43.1	42.9	43.1	43.1	54.0	54.8	54.8	54.8	54.0
JEV_SA14-14-2.D90195.1	5	49.7	46.7	48.0	47.5	99.8	100.0	98.2	98.8	98.0	39.8	76.3	76.7	76.9	43.1	42.9	43.1	43.1	54.0	54.8	54.8	54.8	54.0
JEV_SA14.D90194.1	6	50.1	47.1	48.2	47.7	98.0	98.2	100.0	99.4	99.4	40.0	77.5	77.8	78.0	43.3	43.3	43.3	43.3	54.4	55.4	55.2	55.2	54.4
JEV_virus.M55506.1	7	49.9	46.9	48.2	47.5	98.6	98.8	99.4	100.0	99.2	40.0	77.3	77.6	77.8	43.3	43.3	43.3	43.3	54.2	55.2	55.0	55.0	54.2
JEV_virus.NC_001437.1	8	50.5	47.5	48.6	48.1	97.8	98.0	99.4	99.2	100.0	40.4	78.0	78.4	78.6	43.5	43.5	43.5	43.5	54.8	55.4	55.2	55.2	54.8
TEV_virus.NC_001672.1	9	38.3	38.9	38.2	39.7	39.6	39.8	40.0	40.0	40.4	100.0	41.3	41.6	41.0	42.3	41.9	42.3	42.3	39.9	39.9	39.6	39.6	39.9
WNV_956.NC_001563.2	10	51.5	48.4	46.8	49.6	76.1	76.3	77.5	77.3	78.0	41.3	100.0	94.6	94.0	43.8	43.6	43.8	43.8	55.0	55.0	54.8	54.8	55.0
WNV_Chin-01.AY490240.2	11	50.5	47.6	46.7	49.6	76.5	76.7	77.8	77.6	78.4	41.6	94.6	100.0	98.8	43.9	43.5	43.9	43.9	54.4	54.2	54.0	54.0	54.4
WNV_NY99_isol-385-99.NC_009942.1	12	50.9	47.6	45.3	49.8	76.7	76.9	78.0	77.8	78.6	41.0	94.0	98.8	100.0	43.9	43.5	43.9	43.9	54.0	53.8	53.8	53.8	54.0
YFV_17D_vaccine_strain.NC_002031.1	13	43.0	43.6	41.6	40.0	43.1	43.1	43.3	43.3	43.5	42.3	43.8	43.9	43.9	100.0	97.6	100.0	99.8	42.0	43.4	43.5	43.5	42.0
YFV_ASIB.AY640589.1	14	43.8	44.4	42.2	40.6	42.9	42.9	43.3	43.3	43.5	41.9	43.6	43.5	43.5	97.6	100.0	97.6	97.4	42.4	43.8	43.9	43.9	42.4
YFV_isol-Pasteur_17D-204.X15062.1	15	43.0	43.6	41.6	40.0	43.1	43.1	43.3	43.3	43.5	42.3	43.8	43.9	43.9	100.0	97.6	100.0	99.8	42.0	43.4	43.5	43.5	42.0
YFV_vaccine_strain_17D-213.U17067.1	16	43.2	43.6	41.7	40.0	43.1	43.1	43.3	43.3	43.5	42.3	43.8	43.9	43.9	99.8	97.4	99.8	100.0	42.0	43.4	43.5	43.5	42.0
ZVVBeH818995.KU365777.1	17	57.3	53.6	57.3	55.8	54.0	54.0	54.4	54.2	54.8	39.9	55.0	54.4	54.0	42.0	42.4	42.0	42.0	100.0	96.8	96.4	96.4	99.8
ZVV_MR766-NIID.LC002520.1	18	57.9	53.8	57.7	56.4	54.8	54.8	55.4	55.2	55.4	39.9	55.0	54.2	53.8	43.4	43.8	43.4	43.4	96.8	100.0	98.8	98.8	96.6
ZVV_MR_766.AY632535.2	19	58.4	54.2	58.2	56.4	54.8	54.8	55.2	55.0	55.2	39.6	54.8	54.0	53.8	43.5	43.9	43.5	43.5	96.4	98.8	100.0	100.0	96.2
ZVV_MR_766.NC_012532.1	20	58.4	54.2	58.2	56.4	54.8	54.8	55.2	55.0	55.2	39.6	54.8	54.0	53.8	43.5	43.9	43.5	43.5	96.4	98.8	100.0	100.0	96.2
ZVV_ZikaSPH2015.KU321639.1	21	57.1	53.8	57.1	55.8	54.0	54.0	54.4	54.2	54.8	39.9	55.0	54.4	54.0	42.0	42.4	42.0	42.0	99.8	96.6	96.2	96.2	100.0

50 % Identity and higher
60 % Identity and higher
70 % Identity and higher

Figure 6

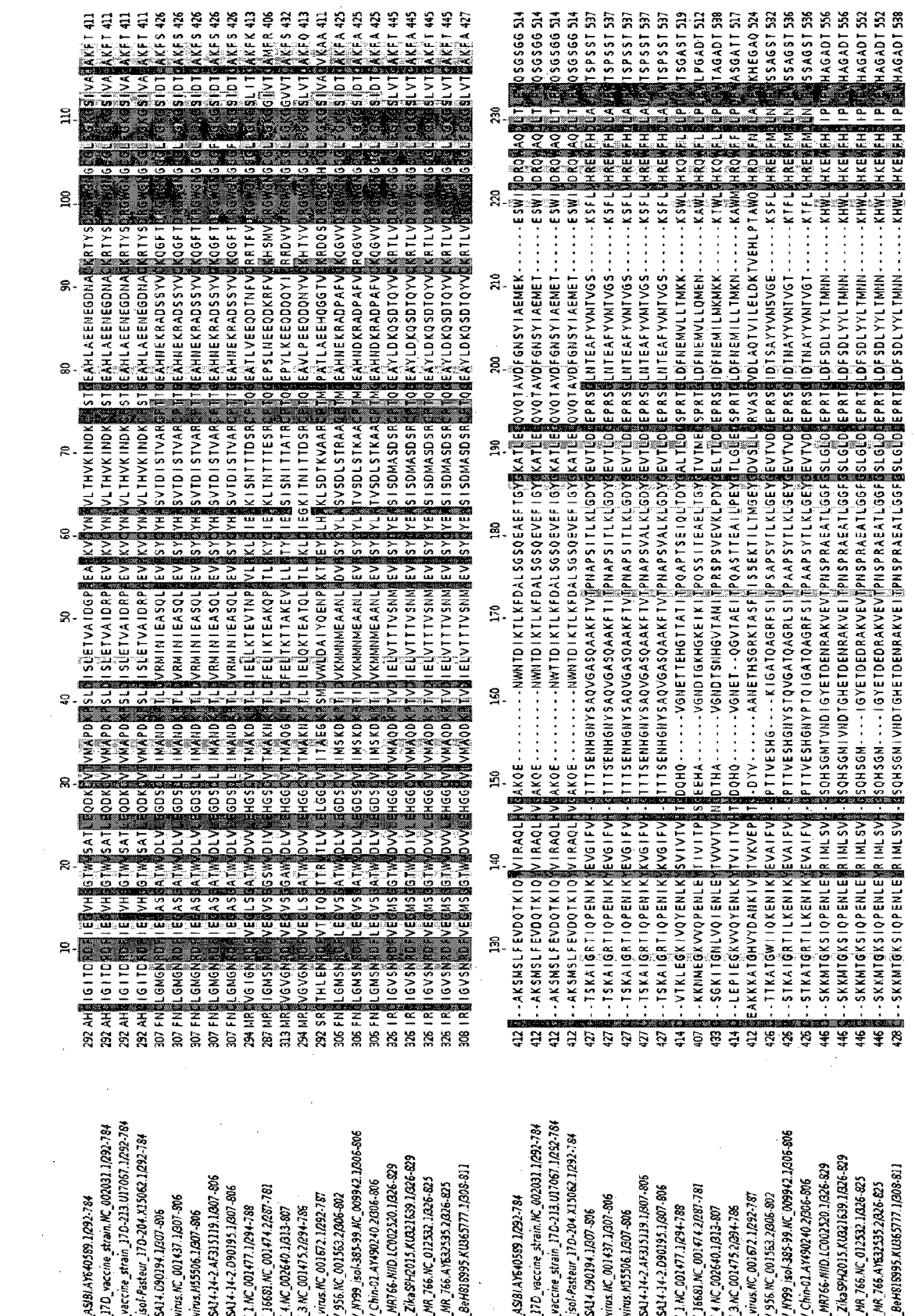


Figure 7A

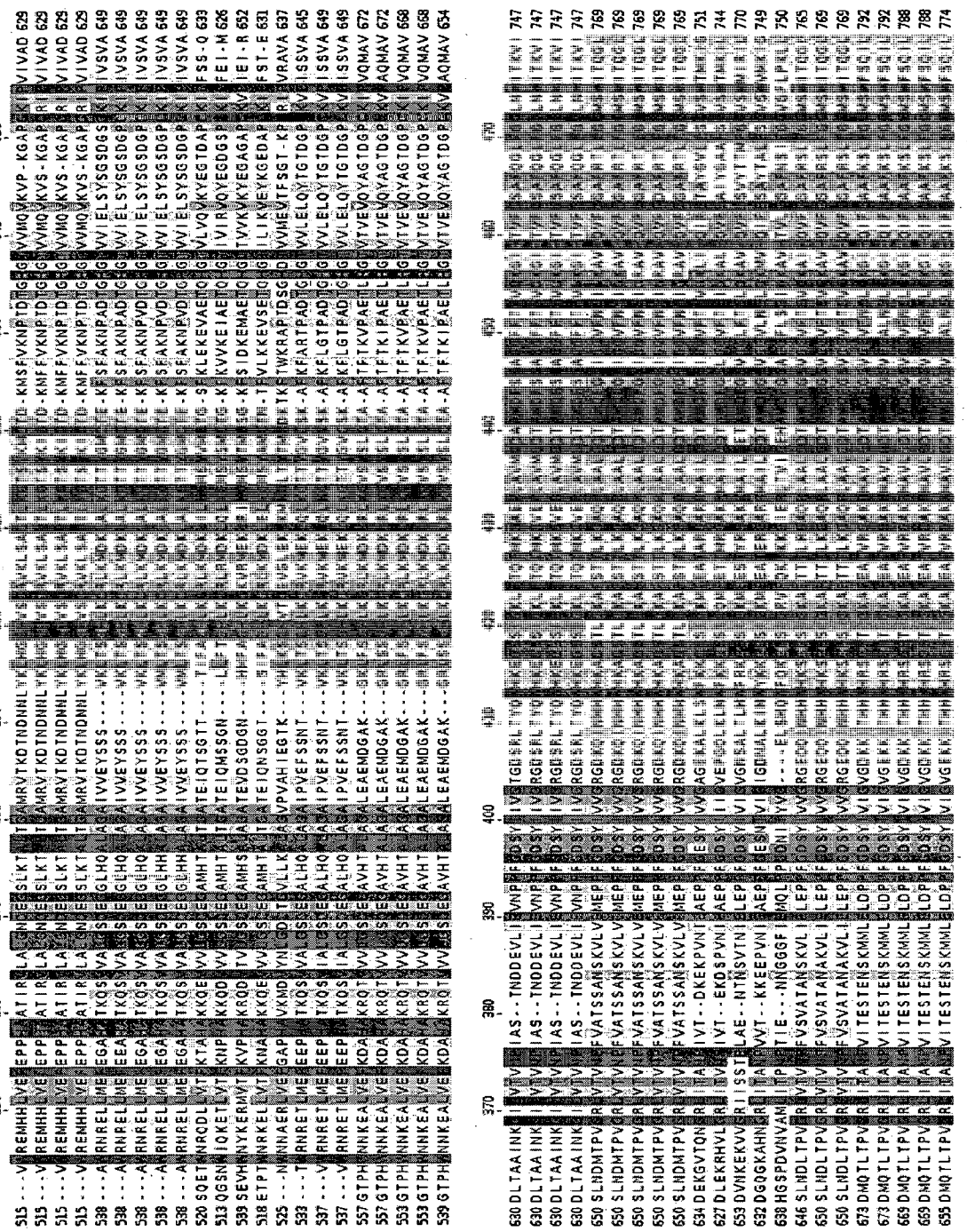


Figure 7B

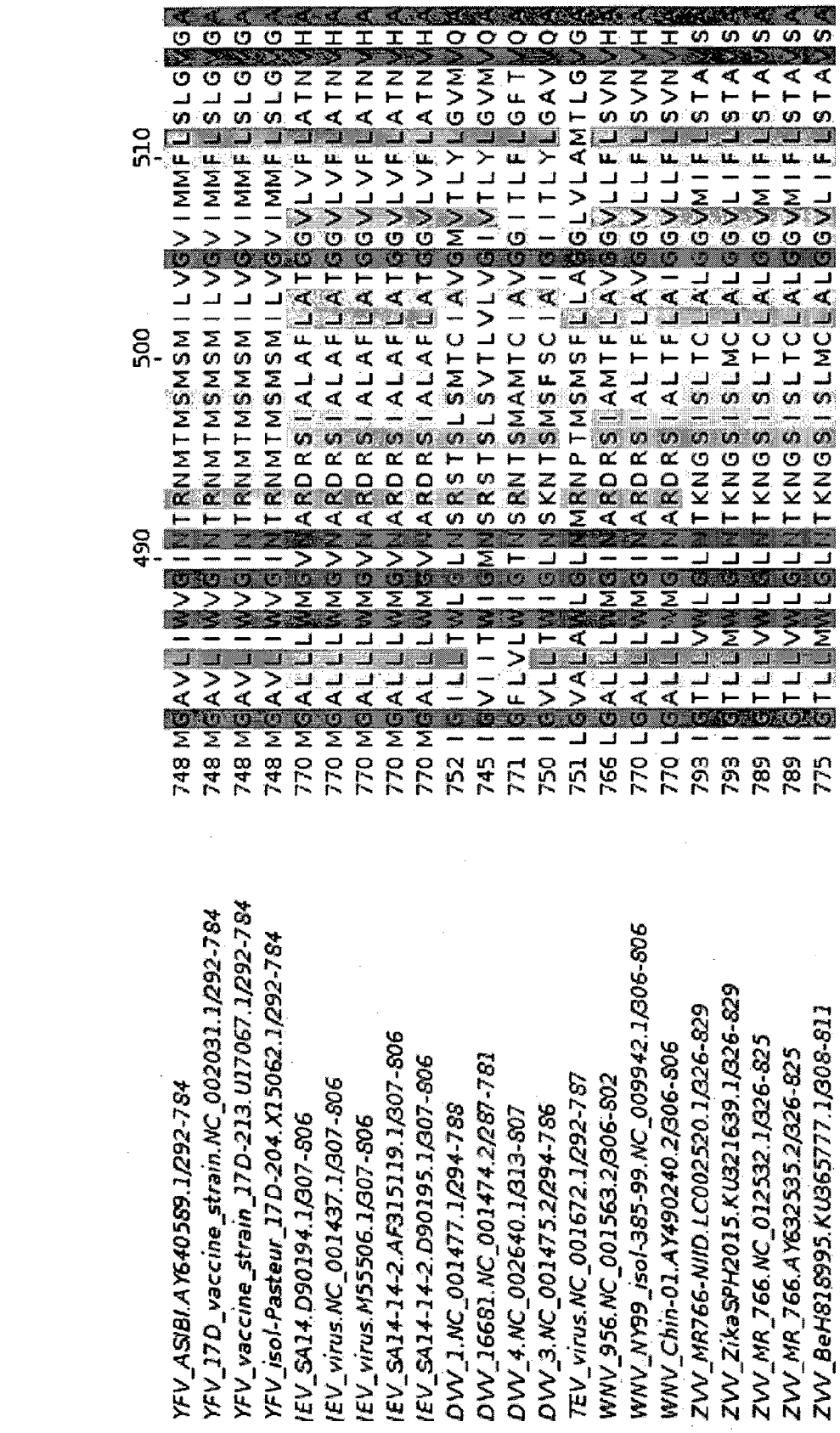

490
500
510
YFV_ASIBI.AY640589.1/292-784
YFV_17D_vaccine_strain.NC_002031.1/292-784
YFV_vaccine_strain_17D-213.U17067.1/292-784
YFV_isol-Pasteur_17D-204.X15062.1/292-784
IEV_SA14.D90194.1/307-806
IEV_virus.NC_001437.1/307-806
IEV_virus.M55506.1/307-806
IEV_SA14-14-2.AF315119.1/307-806
IEV_SA14-14-2.D90195.1/307-806
DVV_1.NC_001477.1/294-788
DVV_16681.NC_001474.2/287-781
DVV_4.NC_002640.1/313-807
DVV_3.NC_001475.2/294-786
TEV_virus.NC_001672.1/292-787
WNV_956.NC_001563.2/306-802
WNV_NY99_isol-385-99.NC_009942.1/306-806
WNV_Chin-01.AY490240.2/306-806
ZVV_MR766-NIID.LC002520.1/326-829
ZVV_ZikaSPH2015.KU321639.1/326-829
ZVV_MR_766.NC_012532.1/326-825
ZVV_MR_766.AY632535.2/326-825
ZVV_BeH818995.KU365777.1/308-811

Figure 7C

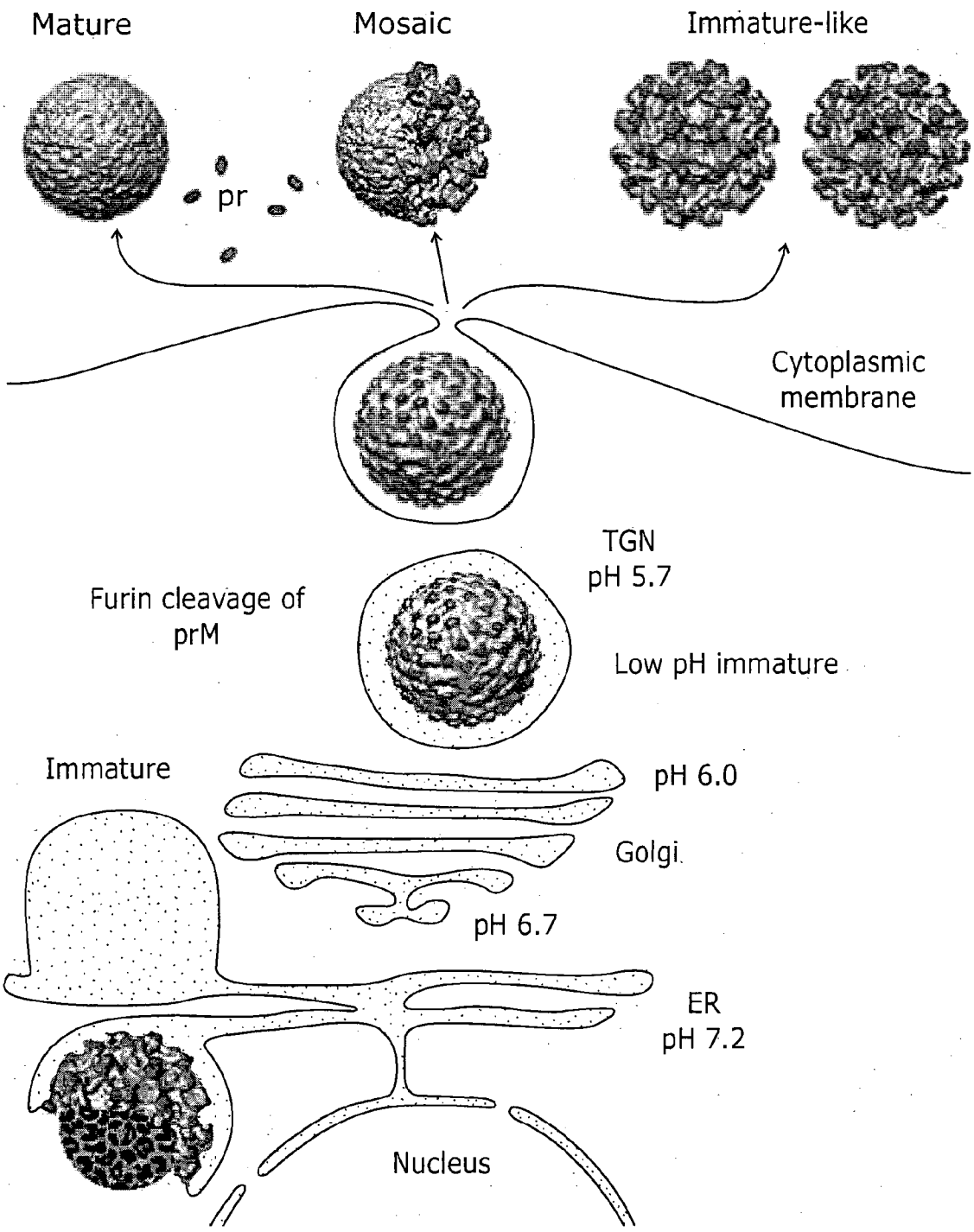

Mature
Mosaic
Immature-like
pr
Cytoplasmic membrane
TGN
pH 5.7
Furin cleavage of prM
Low pH immature
Immature
pH 6.0
Golgi
pH 6.7
ER
pH 7.2
Nucleus

Figure 8

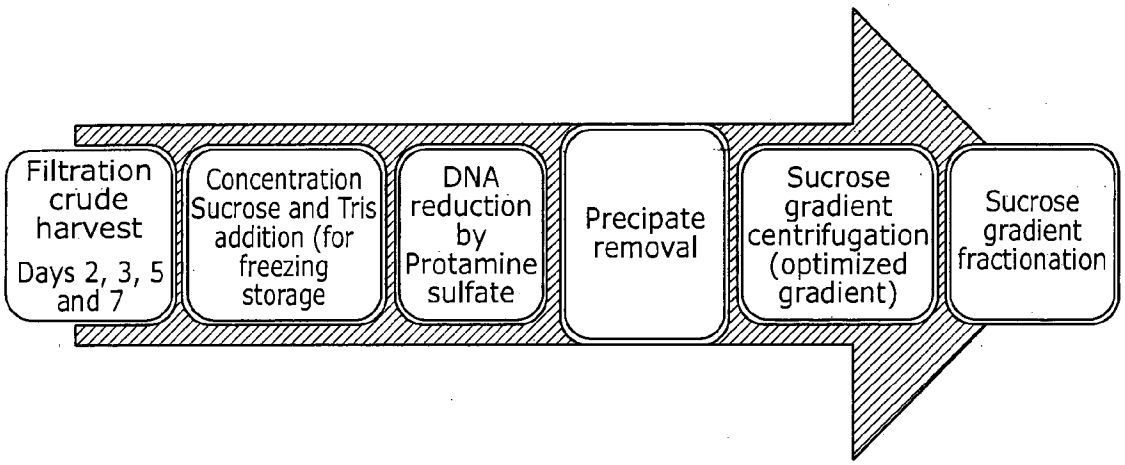


Figure 9A

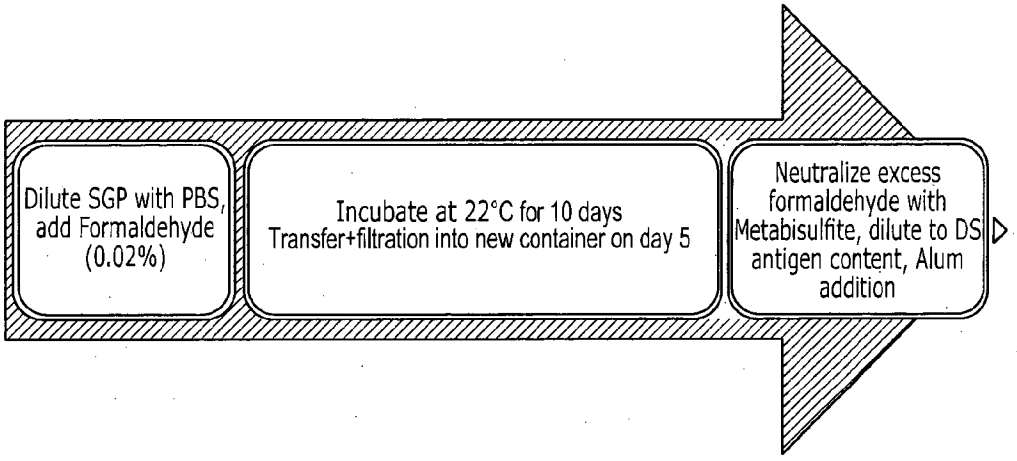


Figure 9B

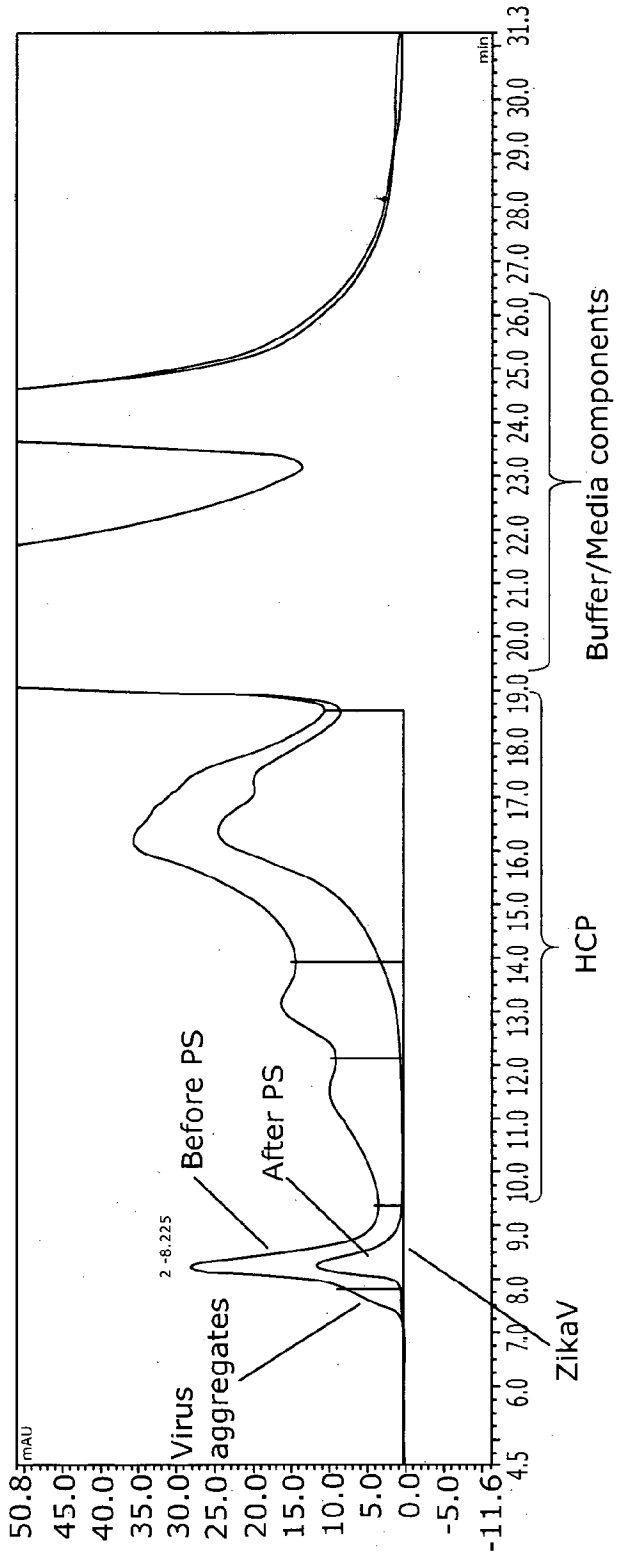
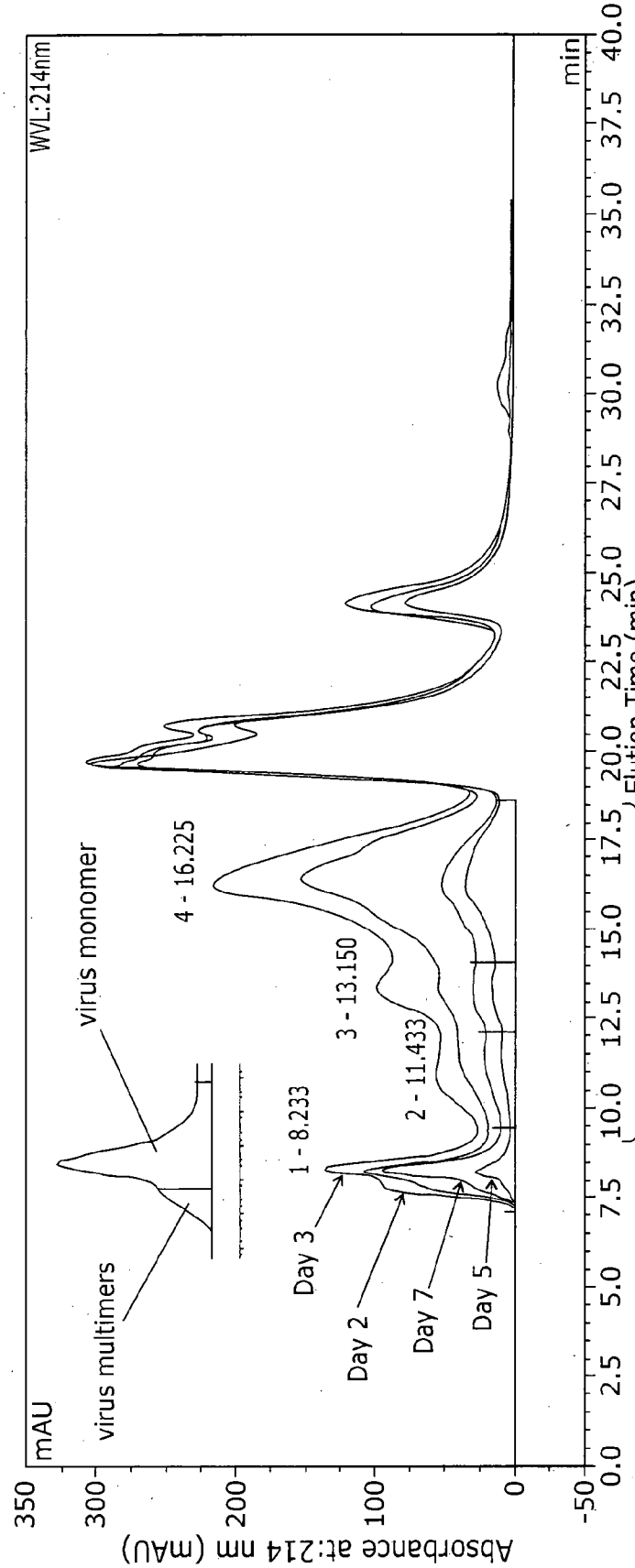

mAU
50.8
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
-11.6
4.5
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0
21.0
22.0
23.0
24.0
25.0
26.0
27.0
28.0
29.0
30.0
31.3
min
2 -8.225
Virus aggregates
Before PS
After PS
ZikaV
HCP
Buffer/Media components

Figure 10



+ Similar virus concentration in harvests Day 2, Day3, Day 7; lower virus concentration in harvest Day 5
+ Day 2 and Day 3 harvests contained more HCP than Days 5/7 (correlates with observed CPE)
+ Minor amount of virus multimers observed

Figure 11

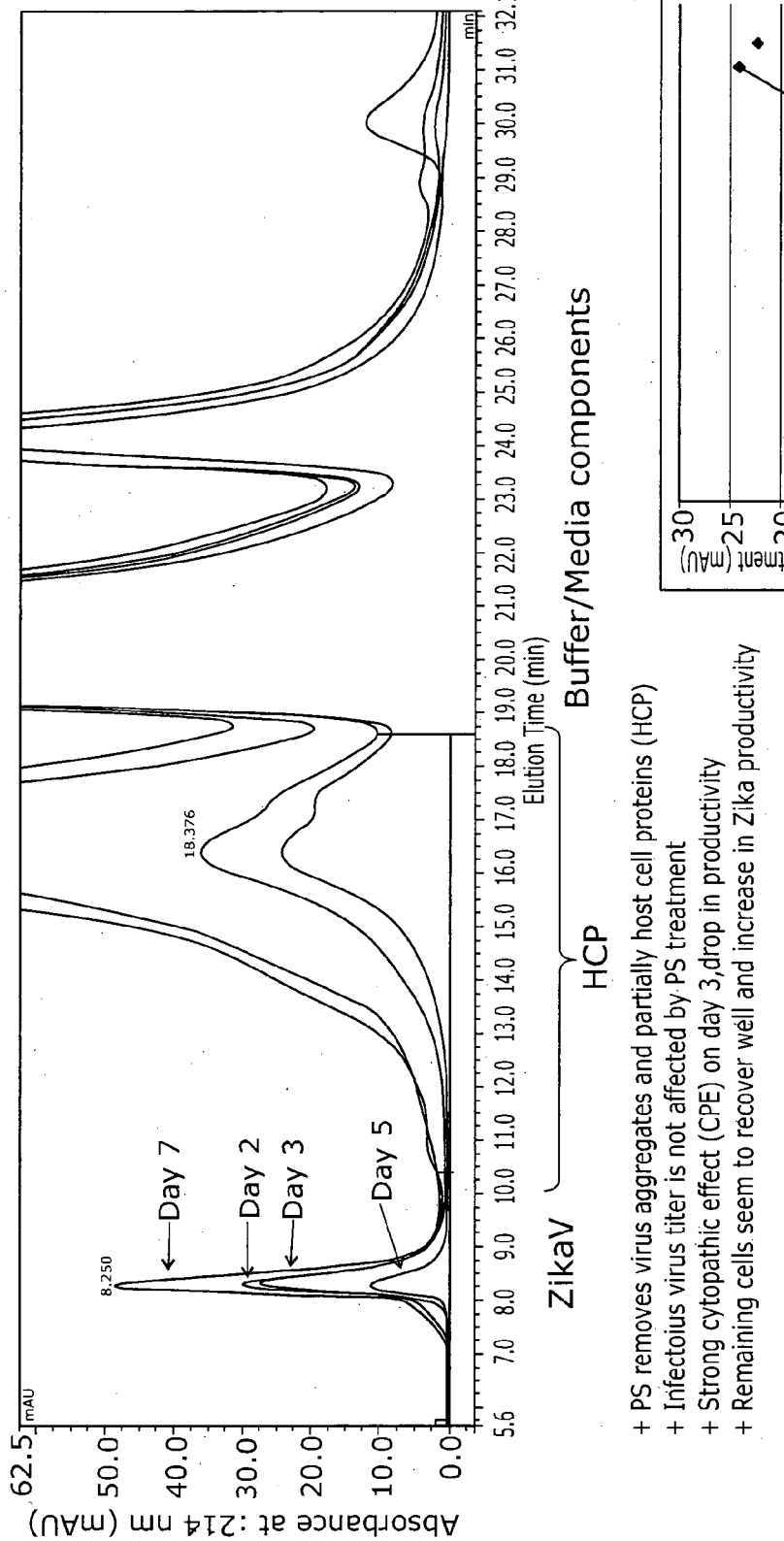

Absorbance at :214 nm (mAU)
mAU
62.5
50.0
40.0
30.0
20.0
10.0
0.0
8.250
18.376
Day 7
Day 2
Day 3
Day 5
5.6 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0 31.0 32.1
min
Elution Time (min)
ZikaV
HCP
Buffer/Media components
+ PS removes virus aggregates and partially host cell proteins (HCP)
+ Infectoius virus titer is not affected by PS treatment
+ Strong cytopathic effect (CPE) on day 3,drop in productivity
+ Remaining cells seem to recover well and increase in Zika productivity
Zika peak Area after PS treatment (mAU)
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8
Days after Infection
CPE observed
Drop in productivity
Cell recovery after CPE
Increase in productivity

Figure 12

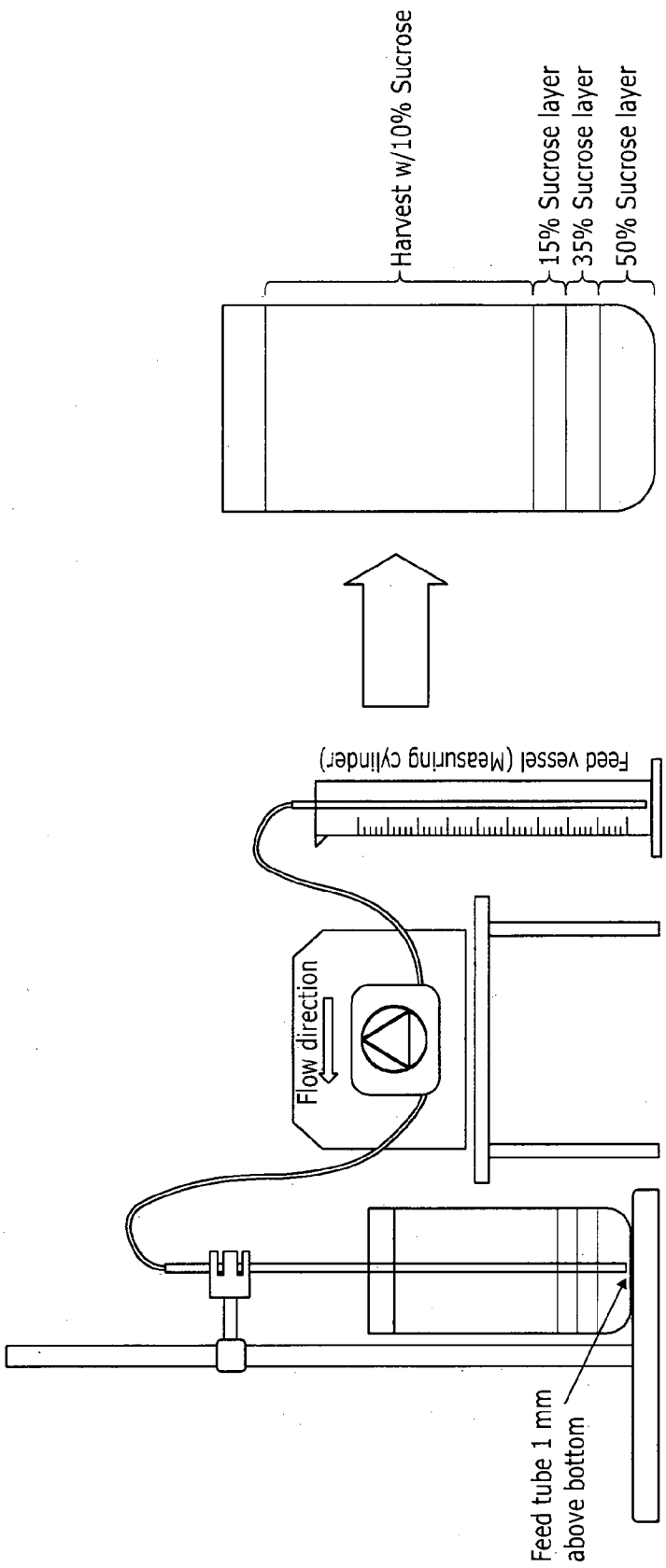
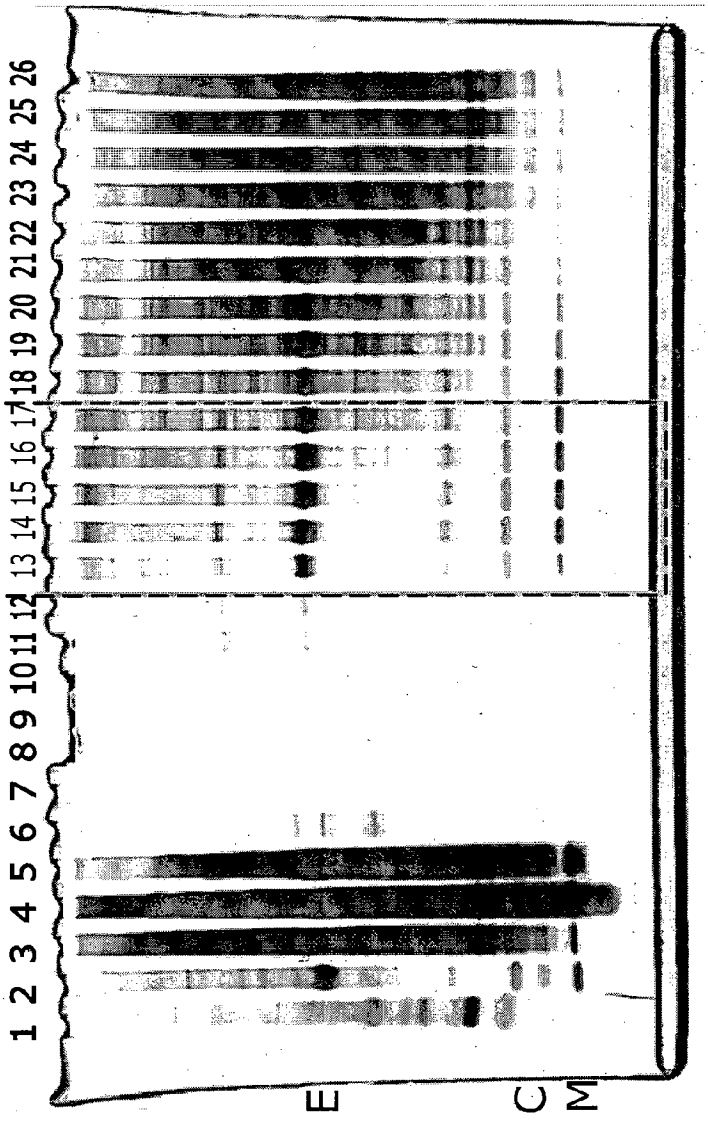

Harvest w/10% Sucrose
15% Sucrose layer
35% Sucrose layer
50% Sucrose layer
Feed vessel (Measuring cylinder)
Flow direction
Feed tube 1 mm above bottom

Figure 13



Lane	Sample
1	Molecular weight Marker
2	JEV Sucrose Gradient Pool (Reference)
3	Zika Harvest pool (day 2,3,5,7)
4	Zika Harvest pool (day 2,3,5,7)concentrated 300x
5	Zika Harvest pool (day 2,3,5,7)concentrated 300x + Protamine sulfate
6	Ultrafiltration Permeate
7	SGC Bottle 1, Fraction 4
8	SGC Bottle 1, Fraction 5
9	SGC Bottle 1, Fraction 6
10	SGC Bottle 1, Fraction 7
11	SGC Bottle 1, Fraction 8
12	SGC Bottle 1, Fraction 9
13	SGC Bottle 1, Fraction 10
14	SGC Bottle 1, Fraction 11
15	SGC Bottle 1, Fraction 12
16	SGC Bottle 1, Fraction 13
17	SGC Bottle 1, Fraction 14
18	SGC Bottle 1, Fraction 15
19	SGC Bottle 1, Fraction 16
20	SGC Bottle 1, Fraction 17
21	SGC Bottle 1, Fraction 18
22	SGC Bottle 1, Fraction 19
23	SGC Bottle 1, Fraction 20
24	SGC Bottle 1, Fraction 21
25	SGC Bottle 1, Fraction 22
26	SGC Bottle 1, Fraction 23

Pooled fractions F10-F14
(10mL total volume pere bottle)

Figure 14

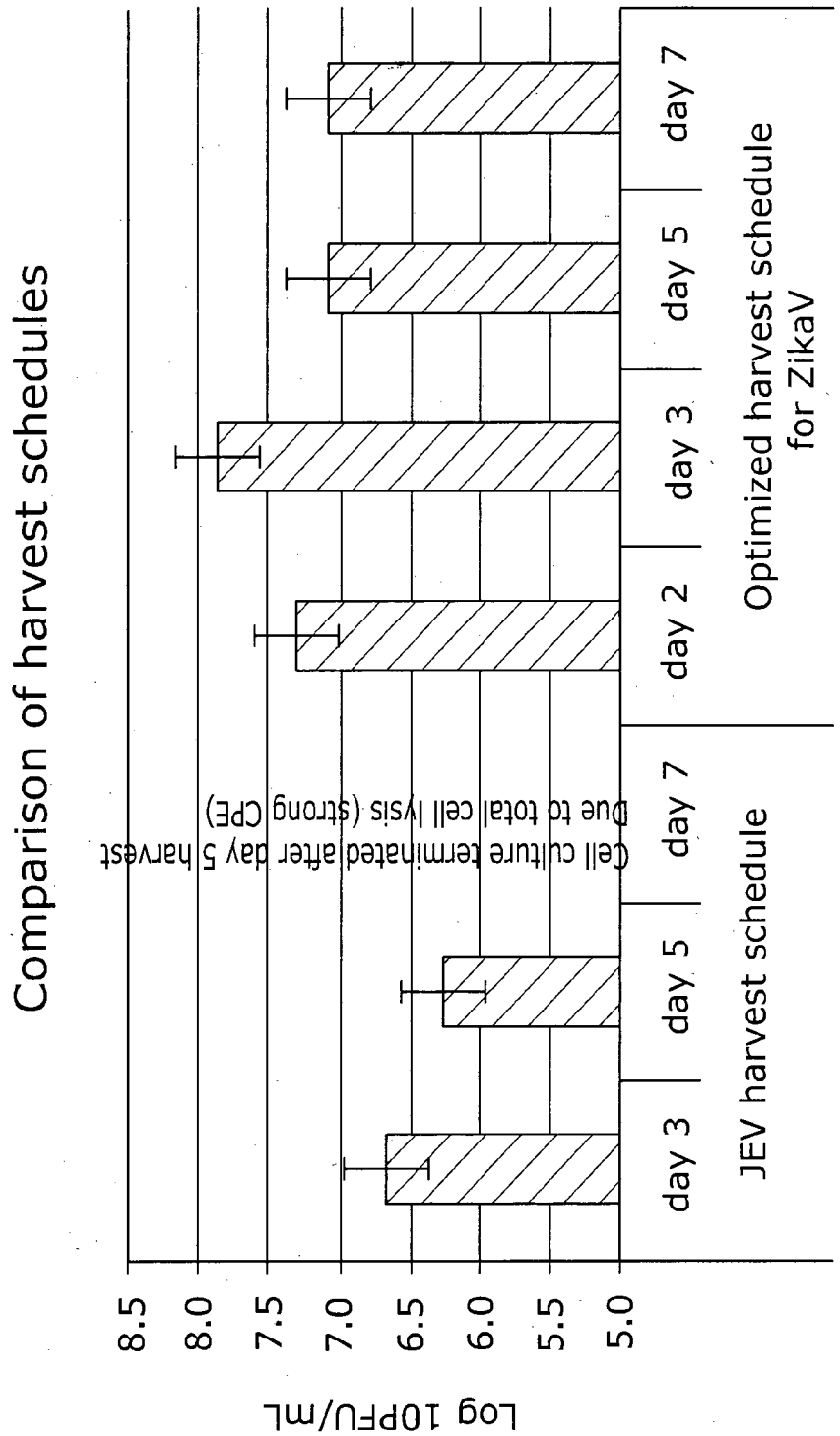

Comparison of harvest schedules
Log 10PFU/mL
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
day 3
day 5
day 7
JEV harvest schedule
day 2
day 3
day 5
day 7
Optimized harvest schedule for ZikaV
Cell culture terminated after day 5 harvest
Due to total cell lysis (strong CPE)

Figure 15

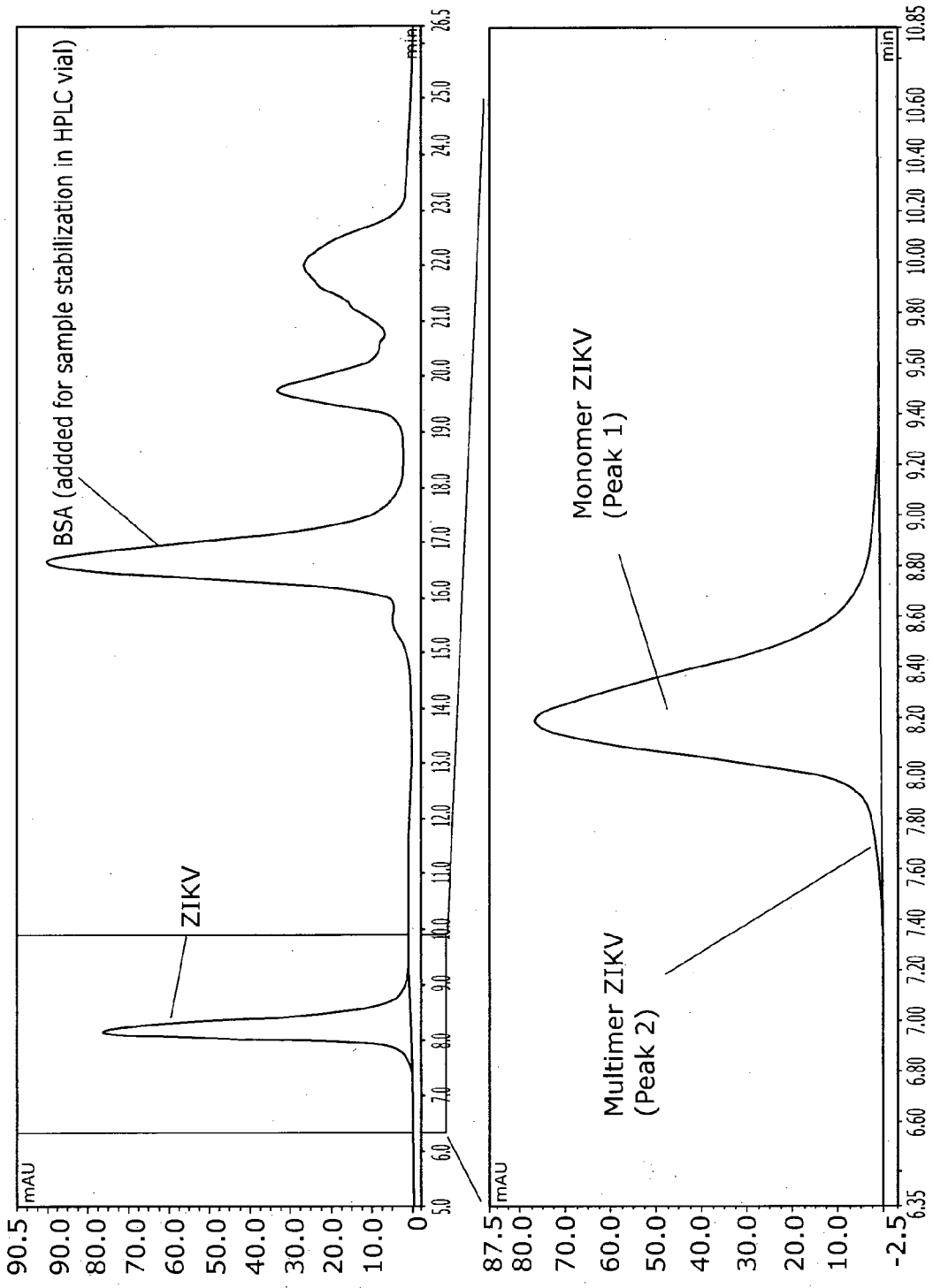

BSA (addded for sample stabilization in HPLC vial)
ZIKV
mAU
min
Monomer ZIKV
(Peak 1)
Multimer ZIKV
(Peak 2)

Figure 16

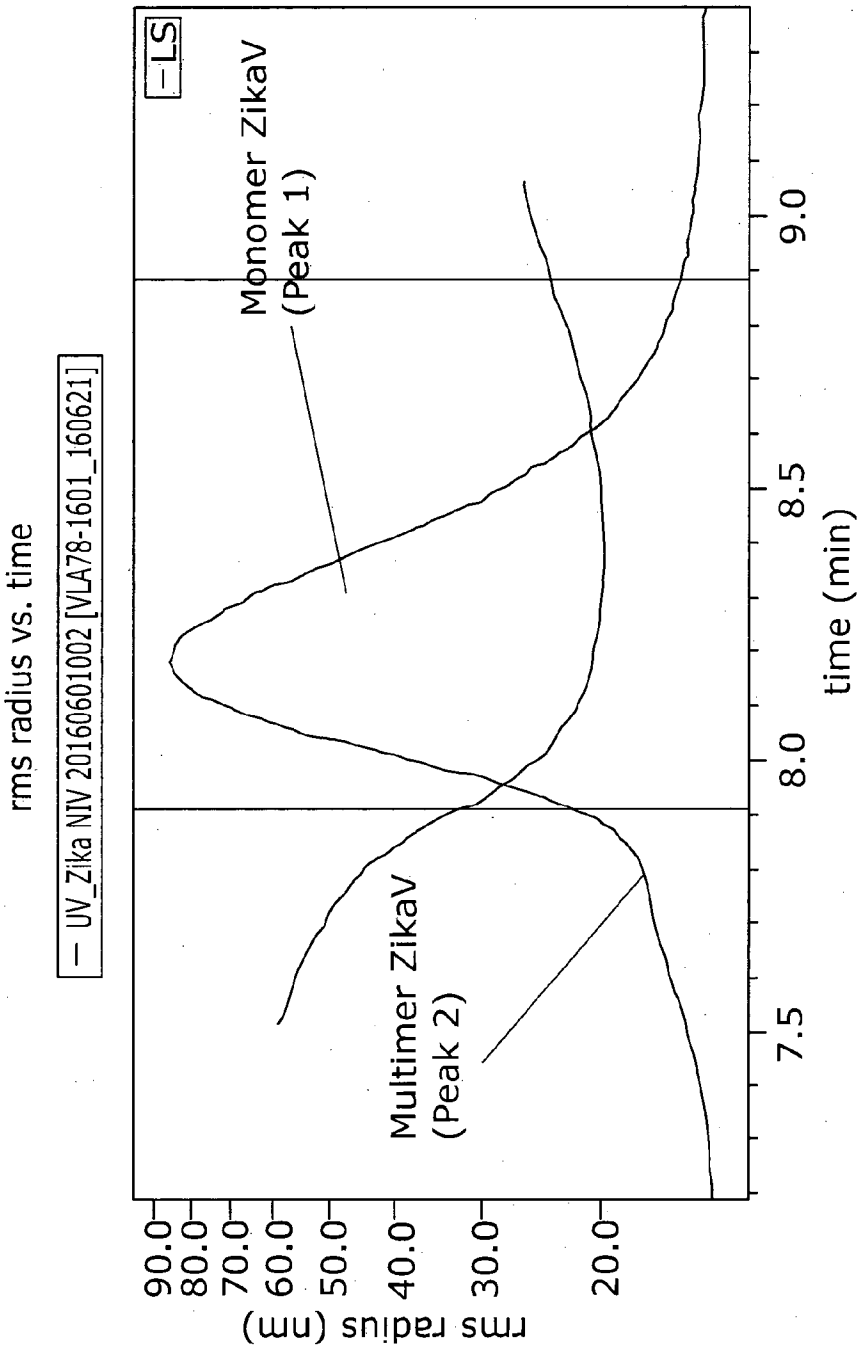

rms radius vs. time
UV_Zika NIV 20160601002 [VLA78-1601_160621]
LS
Monomer ZikaV
(Peak 1)
Multimer ZikaV
(Peak 2)
rms radius (nm)
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
7.5
8.0
8.5
9.0
time (min)

Figure 17

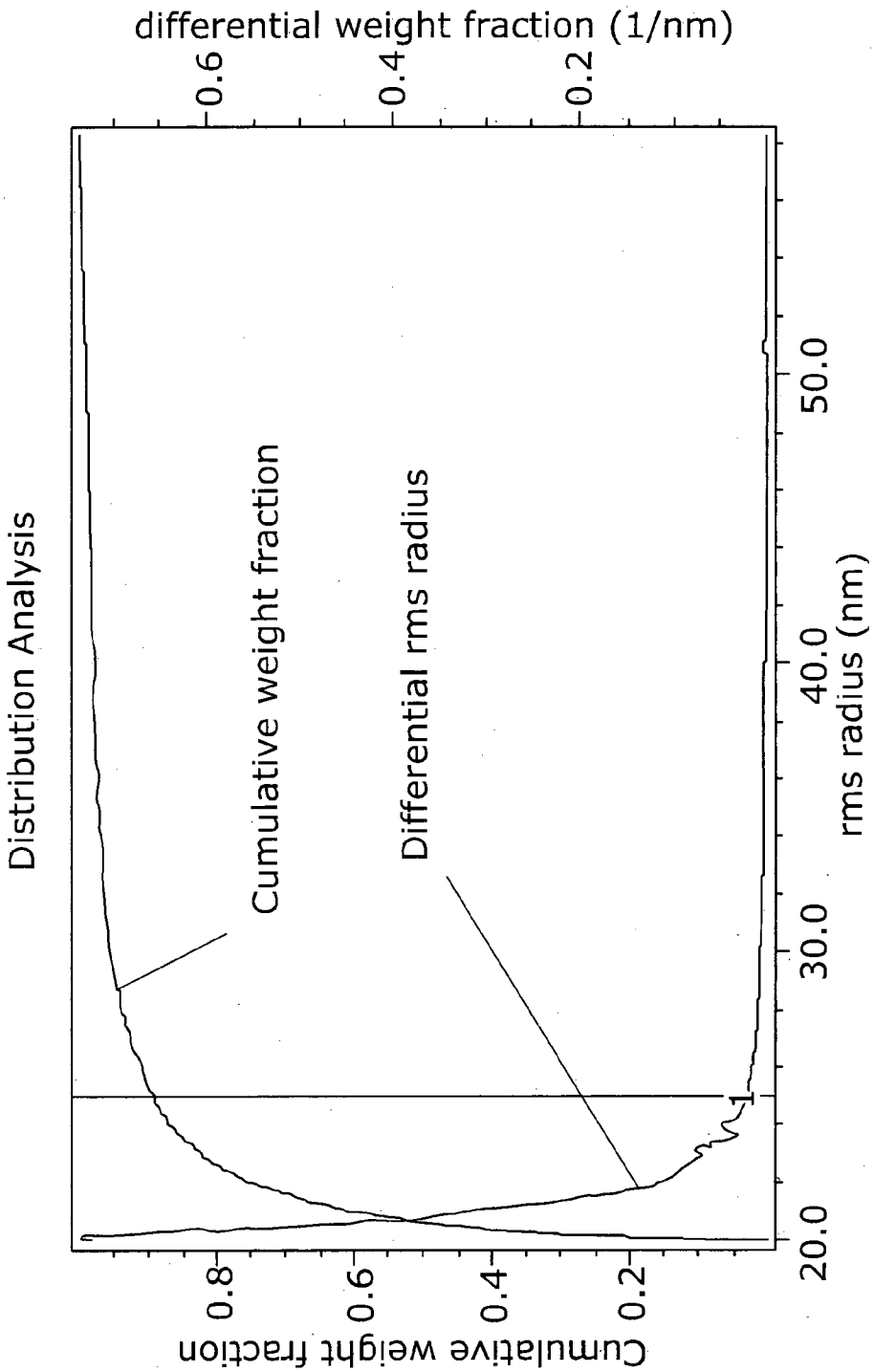
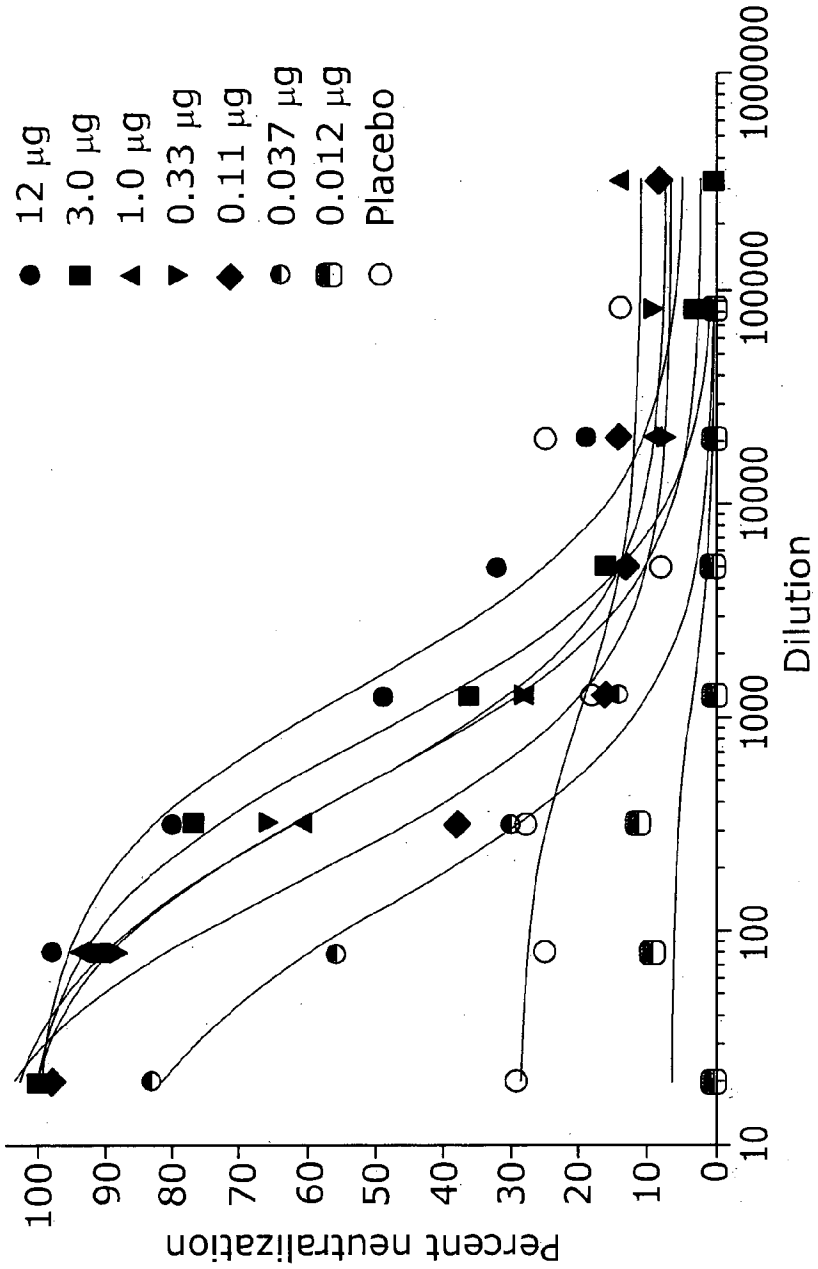
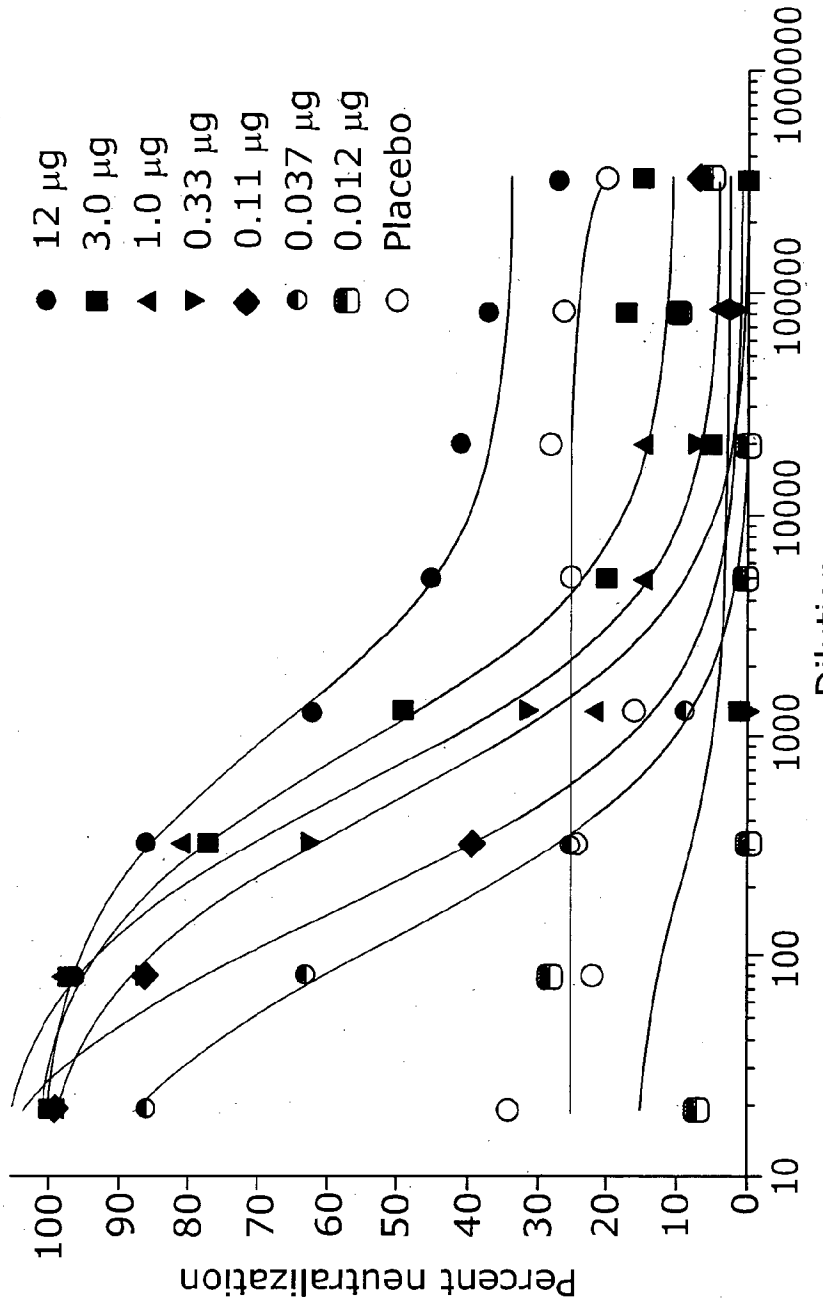

Distribution Analysis
Cumulative weight fraction
Differential rms radius
Cumulative weight fraction
0.8
0.6
0.4
0.2
20.0
30.0
40.0
50.0
rms radius (nm)
differential weight fraction (1/nm)
0.6
0.4
0.2

Figure 18



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1371	807.6	380.7	456.1	179.9	142.0

Figure 19



	12 µg	3.0 µg	1.0 µg	0.33 µg	0.11 µg	0.037 µg
EC50	1029	819.1	556.9	477.3	158.1	118.0

Figure 20

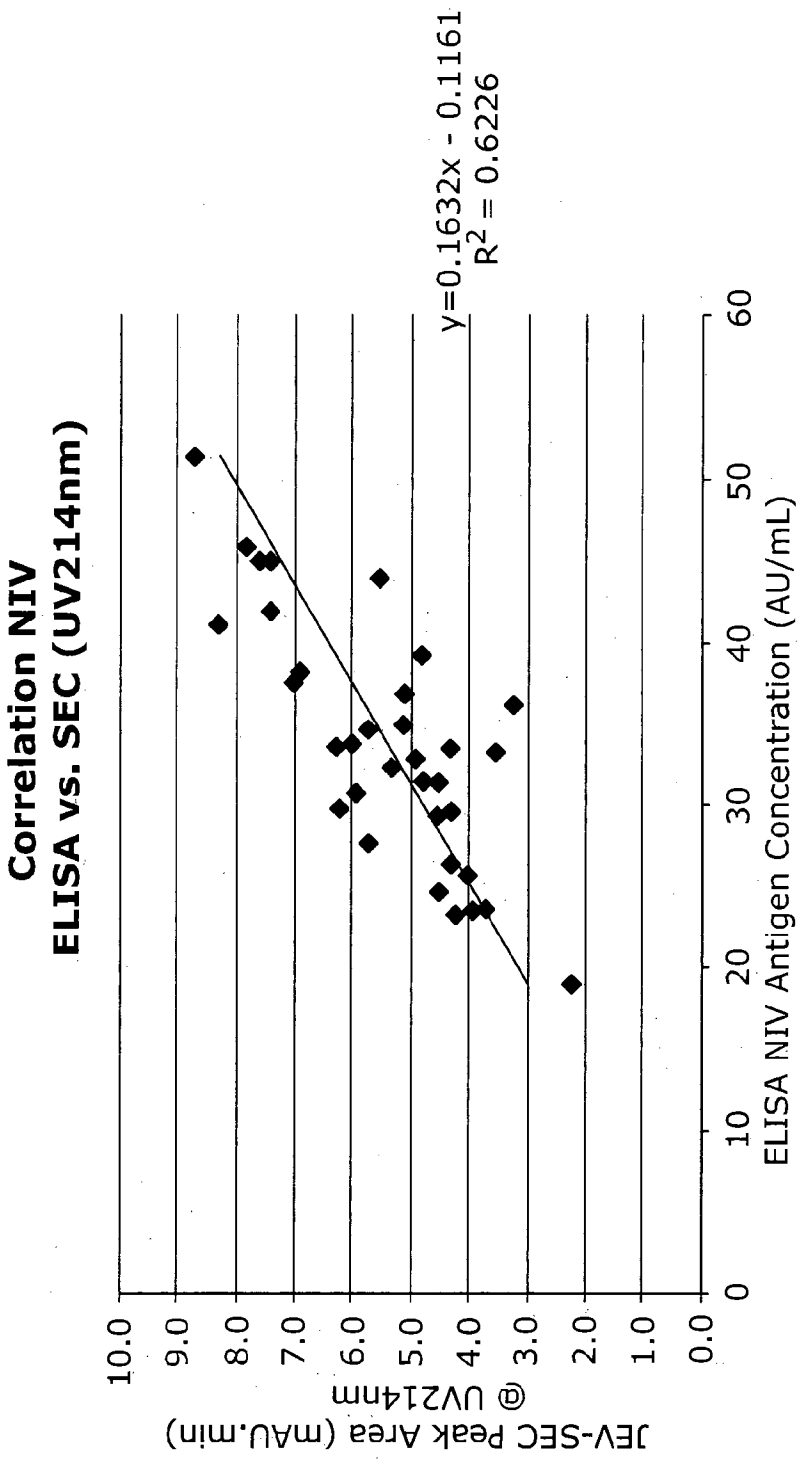

Correlation NIV
ELISA vs. SEC (UV214nm)
y=0.1632x - 0.1161
R2 = 0.6226
JEV-SEC Peak Area (mAU.min)
@ UV214nm
ELISA NIV Antigen Concentration (AU/mL)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
10
20
30
40
50
60

Figure 21

ZIKA VIRUS VACCINE

RELATED APPLICATION

This Application is a national stage filing under 35 U.S.C. § 371 of International Patent Application Serial No. PCT/EP2016/082664, filed December 23, 2016, the contents of which is incorporated herein by reference in its entirety.

FIELD OF INVENTION

The disclosure relates to methods for the purification of Zika viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate. The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central

America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measures involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

A preventative vaccine against Zika virus is a pressing medical need in endemic areas and in geographical areas where the vector is spreading. Furthermore, as Zika infection has dire consequences on embryonic and fetal development, a safe and effective vaccine for women of child-bearing potential or pregnant women is needed. Vaccines administered during pregnancy must be very safe for both the mother and the developing fetus. While live attenuated viral vaccines are highly effective, they are often not considered safe enough for administration to pregnant women. In this regard, inactivated viral vaccines, which lack the ability to propagate in the vaccinated subject, are considered much safer. Development of an inactivated Zika virus vaccine for administration to at-risk patients would fill this need.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can be very efficiently separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being

exposed to Zika virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Detail experimental examples to the above are provided for Zika virus.

The herein disclosed in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was unexpectedly higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, Nature doi:10.1038/nature18952.). Inactivated viruses are among the safest vaccines and especially preferred for delivery to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figures 7A-7C ~~**Figure 7**~~: Alignment (shading: % identity, aa), E-protein.

Figure 8: An example of virus particle maturation in the host cell. As observed in flaviviruses, full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID50 activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 9: An exemplary downstream virus purification process from the crude harvest to formulation of the drug substance (vaccine), a preferred embodiment of the process of the invention (**A**). A flow-chart of an exemplary virus inactivation process is shown in (**B**).

Figure 10: PS treatment results in selective removal of Zika virus aggregates and Vero HCP and LMW impurities (SEC-HPLC of 30x concentrated Zika Virus harvest day 5).

Figure 11: SEC-HPLC of individual 30x concentrated Zika harvest prior to PS treatment at different time points.

Figure 12: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment at different time points. The smaller graph indicates the observed cytopathic effect (CPE) over time.

Figure 13: Preparation of the sucrose gradient.

Figure 14: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 15: Comparison of JEV and ZikaV harvest schedules/yields.

Figure 16: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column. Both panels are from the same chromatogram. The upper graph is the complete elution profile; the lower graph is an enlargement of the ZIKAV elution peak.

Figure 17: SEC-MALLS analysis of inactivated ZikaV.

Figure 18: Cumulative particle size distribution of Zika NIV.

Figure 19: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 20: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 21: Correlation between JEV antigen content in neutralized inactivated virus (NIV) analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in

particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus.

Disclosed herein are downstream processes for purifying Zika virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for propagation on a particular cell substrate or for a partially / fully cell substrate adapted Zika virus particle.

Aspects of the invention provide processes for the purification of infectious Zika virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the content of residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL.

In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the

detection limit of HPLC, in particular below the detection limit in the final drug substance. In some embodiments, the PS content is tested by HPLC or size exclusion chromatography (SEC). For example, HPLC is validated for PS determination in JEV sucrose gradient pool samples as a routine release assay and is very sensitive (i.e., LOQ 3 µg/mL; LOD 1 µg/mL). In the current invention, PS content in in Zika virus DS samples was <LOD. In one embodiment, the HPLC assessment of PS content can be performed on a Superdex Peptide 10/300GL column (GE: 17-5176-01) using 30% Acetonitrile, 0,1% Trifluoroacetic acid as solvent with a flow rate of 0,6 ml/min at 25°C and detection at 214 nm. A more sensitive method of measurement for residual protamine in a purified virus preparation is mass spectrometry (MS). In some embodiments, the residual PS levels in a Zika virus preparation are tested by MS or other such highly sensitive method, e.g. nuclear magnetic resonance (NMR). With this method, residual PS, as well as fragments and/or break-down products of PS, can be detected at trace amounts, such as levels as low as, for example, 10^6, 10^7 or 10^8 molecules per typical sample load. In some embodiments, the PS levels are tested in the sucrose gradient pool. In some embodiments, the PS levels are tested in the drug product. In some embodiments, the PS levels are tested in the drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of about 100 kDa.

The process according to the current invention may also comprise the use of a sucrose gradient, preferably an optimized sucrose gradient. The sucrose gradient is preferably optimized for the removal of protamine sulfate, also for the removal of immature viral particles or other viral particles which are non-infectious or host cell proteins or nucleic acids (DNA, RNA, mRNA, etc) or other host cell debris. In the current invention the optimized sucrose gradient comprises at least two, at least three, at least four layers of sucrose solutions with different densities. In one embodiment, the virus

preparation to be purified is provided in a sucrose solution which has a density of about 8%, about 9%, about 10%, about 11%, about 12% sucrose (w/w), preferably about 10%. In one embodiment, one sucrose solution in the gradient has a density of about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55% sucrose (w/w), preferably about 50%. In one embodiment, one sucrose solution in the gradient has a density of about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40% sucrose (w/w), preferably about 35%. In one embodiment, one sucrose solution in the gradient has a density of about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20% sucrose (w/w), preferably about 15% sucrose. In a preferred embodiment, the sucrose gradient comprises three layers of sucrose solutions of about 50%, about 35% and about 15% (w/w) sucrose and the virus composition to be purified is contained in about 10% (w/w) sucrose. Because the invention provided for means to not only test for host cell DNA but also immature viral particles, the skilled person in the art is able to more precisely optimize the sucrose gradient for most efficient purification and include additional tools such as PRNT assay to monitor purification success.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may be optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the Zika virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In preferred embodiments, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a

chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian cell line. In some embodiments, said cell line is an EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the Zika virus particles obtainable by any of the processes described herein for treating and/or preventing (i.e. protecting from) a viral infection. In a preferred embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on

the average for their age, sex, and ethnicity. This is a result of the brain failing to undergo proper embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has an RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the process of the invention results in an enrichment of infectious Zika virus particles from the crude harvest comprising infectious Zika virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%, especially at least 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

A unique aspect of the current invention is the realization that know-how related to the vaccine design and purification approach used for the Japanese Encephalitis Vaccine (JEV) IXIARO® (see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) may be employed and improved upon in order to expedite the development of a Zika virus vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, was complemented by further significant improvements disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines is their greatly reduced protamine salt (SEQ ID NO: 1) content in the final drug substance facilitated by the development of an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the Vero (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or the plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently

inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation needs to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0.01 MOI (multiplicity of infection) to allow for viral production prior to passaging. In some embodiments, the Vero cells are incubated for at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. In order to minimize the adaption of Zika virus strain to Vero cells, it was found that Vero cells could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days

2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 µm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the Zika virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant", is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA. The

fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in the experimental part. It was surprisingly found that the addition of a virus-containing fraction with 10% (w/w) sucrose to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) resulted in efficient separation of protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, i.e. a Zika virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agents for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using

such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphobetaine. A preferred inactivation is inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of

time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments, the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time. It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

Any of the methods or uses described herein may be for the prevention of a Zika virus infection in a subject. As used herein, the terms "prevent," "preventing" and "protection from" include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the Zika virus. As used herein, antigen(s), such as an inactivated Zika virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck, trunk, upper arms; headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject has been previously infected with or vaccinated against Dengue virus; i.e., at risk for antibody-dependent enhancement of disease. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus antibodies). In some embodiments, the therapeutically effective amount is sufficient to provide seroprotection in a subject; i.e., to generate sufficient antigen-specific antibodies to prevent/protect

from infection. In some embodiments, seroprotection is conferred on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects. In some embodiments, seroprotection is defined by a reduction in the number of Zika virus plaques by 50% or more in a plaque reduction neutralization test (PRNT) by a 1:10 or higher dilution of sera from a vaccinated subject. In some embodiments, an effective amount of the Zika vaccine is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the

Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)). Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 71). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its entirety). In some embodiments, the adjuvant is IC31®, i.e. $KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccines to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may

have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having,", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Table 1. Overview of process buffers and stock solutions.

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.
C	25 mM Tris, 150 mM NaCl	7.4±0.2	16.5

D	1 M Tris	7.4±0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
K	10 x PBS	7.4±0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4±0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4±0.2	1.3

Table 2. Abbreviations.

°Bx	Degrees Brix = sugar content (w/w) of an aqueous solution*
BSA	Bovine serum albumin
CC700	Capto™ Core 700
CPE	Cytopathic effect
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
ID	Inner diameter
JEV	Japanese Encephalitis virus
LAL	Limulus amebocyte lysate
LDS buffer	Lithium dodecyl sulfate sample loading buffer
LOD	Limit of detection
LOQ	Limit of quantitation
MALLS	Multiangle light scattering
mAU	Milli absorbance units
MS	Mass spectroscopy
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
PD	Process development
PFU	Plaque forming units
p.i.	Post-infection
PS	Protamine sulphate or protamine sulfate
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin
Rms radius	Root mean square radius
rMSB	Research master seed bank
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SGP	Sucrose gradient purified
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%

UF/DF	Ultrafiltration/diafiltration
WFI	Water for injection
ZikaV	Zika virus

*Degrees Brix (°Bx) is the sugar content of an aqueous solution. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. °Bx corresponds to the sucrose content in percent (w/w), e.g., 45 °Bx equals 45 % (w/w) sucrose.

Table A. Primers for Zika virus sequencing: lower case letters indicate bases not included in ZIKA but containing restriction sites for later cloning when needed (therefore, two Tms provided).

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
1	9320_Zika_PF_1F	SEQ ID NO: 74 ttaggatccGTTGTTGATCTGTGTGAAT	69.9	74.6	707
	9321_Zika_PF_1R	SEQ ID NO: 75 taactcgagCGTACACAACCCAAGTT	69.3	75.6	
2	9322_Zika_PF_2F	SEQ ID NO: 76 ttaggatccTCACTAGACGTGGGAGTG	70	73.9	704
	9323_Zika_PF_2R	SEQ ID NO: 77 taactcgagAAGCCATGTCYGATATTGAT	69.8	73.7	
3	9324_Zika_PF_3F	SEQ ID NO: 78 ttaggatccGCATACAGCATCAGGTG	72.3	74.5	712
	9325_Zika_PF_3R	SEQ ID NO: 79 taactcgagTGTGGAGTTCCGGTGTCT	72	76.4	
4	9326_Zika_PF_4F	SEQ ID NO: 80 ttaggatccGAATAGAGCGAARGTTGAGATA	70.9	74	712
	9327_Zika_PF_4R	SEQ ID NO: 81 taactcgAGTGGTGGGTGATCTTCTTCT	70.5	73.7	
5	9328_Zika_PF_5F	SEQ ID NO: 82 ttaggatcCAGTCACAGTGGAGGTACAGTAC	70.3	75	704
	9329_Zika_PF_5R	SEQ ID NO: 83 taactcgagCRCAGATACCATCTTCCC	71.5	77.3	
6	9330_Zika_PF_6F	SEQ ID NO: 84 ttaggatCCCTTATGTGCTTGGCCTTAG	70.7	72.7	698
	9331_Zika_PF_6R	SEQ ID NO: 85 taactcgagTCTTCAGCCTCCATGTG	70.4	76.9	
7	9332_Zika_PF_7F	SEQ ID NO: 86 ttaggatccAATGCCCACTCAAACATAGA	71.9	75	716
	9333_Zika_PF_7R	SEQ ID NO: 87 taactcgagTCATTCTCTTCTTCAGCCCTT	71	74	
8	9334_Zika_PF_8F	SEQ ID NO: 88 ttaggatccAAGGGTGATCGAGGAAT	70.9	75.2	703
	9335_Zika_PF_8R	SEQ ID NO: 89 taactcgagTTCCCTTCAGAGAGAGGAGC	71.9	73.4	
9	9336_Zika_PF_9F	SEQ ID NO: 90 ttaggatccTCTTTTGCAAACTGCGATC	71.9	75	699
	9337_Zika_PF_9R	SEQ ID NO: 91 taactcgagTCCAGCTGCAAAGGGTAT	71	74.9	
10	9338_Zika_PF_10F	SEQ ID NO: 92 ttaggatccGTGTGGACATGTACATTGA	71.4	75.8	706
	9339_Zika_PF_10R	SEQ ID NO: 93 taactcgagCCCATTGCCATAAAGTC	70.4	75.8	
11	9340_Zika_PF_11F	SEQ ID NO: 94 ttaggatccTCATACTGTGGTCCATGGA	71.6	78.1	692
	9341_Zika_PF_11R	SEQ ID NO: 95 taactcgagGCCCATCTCAACCCTTG	74	78	
12	9342_Zika_PF_12F	SEQ ID NO: 96 ttaggatccTAGAGGGCTTCCAGTGC	70.9	74	707

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
	9343_Zika_PF_12R	SEQ ID NO: 97 taactcgAGATACTCATCTCCAGGTTTGTTG	70.2	72.2	
13	9344_Zika_PF_13F	SEQ ID NO: 98 ttaggatccGAAAACAAAACATCAAGAGTG	70.6	75.4	726
	9345_Zika_PF_13R	SEQ ID NO: 99 taactcgagGAATCTCTCTGTCATGTGTCCT	71.9	75.6	
14	9346_Zika_PF_14F	SEQ ID NO: 100 ttaggatccTTGATGGCACGACCAAC	73.1	75.6	715
	9347_Zika_PF_14R	SEQ ID NO: 101 ttaggatccGTTGTTGATCTGTGTGAAT	70.8	77.9	
15	9348_Zika_PF_15F	SEQ ID NO: 102 taactcgagCAGGTCAATGTCCATTG	71.9	75.4	719
	9349_Zika_PF_15R	SEQ ID NO: 103 ttaggatccTGTTGTGTTCCTATTGCTGGT	73.9	77.2	
16	9350_Zika_PF_16F	SEQ ID NO: 104 taactcgaGTGATCAGRGCCCCAGC	72.3	75.4	703
	9351_Zika_PF_16R	SEQ ID NO: 105 ttaggatccTGCTGCCCAGAAGAGAA	72	76.3	
17	9352_Zika_PF_17F	SEQ ID NO: 106 taactcgaGCACCAACAYGGGTTCTT	73.6	76	705
	9353_Zika_PF_17R	SEQ ID NO: 107 ttaggatcCTCAAGGACGGTGTGGC	72	75.5	
18	9354_Zika_PF_18F	SEQ ID NO: 108 taactcgagCAATGATCTTCATGTTGGG	71.7	75.8	699
	9355_Zika_PF_18R	SEQ ID NO: 109 ttaggatccTATGGGGGAGGACTGGT	71	74.1	
19	9356_Zika_PF_19F	SEQ ID NO: 110 taactcGAGCCCAGAACCTTGGATC	73.3	75.5	711
	9357_Zika_PF_19R	SEQ ID NO: 111 ttaggatcCAGACCCCCAAGAAGGC	71.3	76.9	
20	9358_Zika_PF_20F	SEQ ID NO: 112 taactcgagCCCCTTTGGTCTTGTCT	71.7	75	706
	9359_Zika_PF_20R	SEQ ID NO: 113 ttaggatccAGGAAGGATGTATGCAGATG	71.9	73.9	
21	9360_Zika_PF_21F	SEQ ID NO: 114 taactcgagACATTTGCGCATATGATTTTG	70.4	75.7	709
	9361_Zika_PF_21R	SEQ ID NO: 115 ttaggatccAGGAAGGACACACAAGAGT	71.8	75	
22	9362_Zika_PF_22F	SEQ ID NO: 116 taactcgagACAGGCTGCACAGCTTT	70	79.1	581
	9363_Zika_PF_22R	SEQ ID NO: 117 ttaggatccTCTCTCATAGGGCACAGAC	74.8	81.1	

SEQUENCES

SEQ ID NO: 1
A typical form of protamine
PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 2
KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome

GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTGG
ATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACG
CGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGG
ATGGTCTTGGCAATTCTAGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTG
GGGAAAAAAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGA
AGGAGAAGAAGAGACGGGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGG
TCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACGATGCTGGGGAGGCCATATCTTTTCCAACCACATTG
GGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCT
GGATGAGGGGGTGGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCAT
CACAAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGT
CGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTC
GCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCT
GATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGG
GTTGATATTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTAC
AACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCT
GCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGC
TGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGTCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCG
GGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGT
TAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACC
CTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATG
AATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAAC
TCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGG
AGTCAAGAAGGAGCAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCT
CTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCA
CATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTG
CAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCA
CTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGG
GAGAAGAAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTGAAGCCACTGTGAGAGGTGCCA
AGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCTCAACTCATTGGGCAAGGGCAT
CCATCAAATTTTTGGAGCAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACAAATTCTCATTGGAACGTTGCTG
ATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGGGGGAGTGTTGATCTTCTTATCC
ACAGCCGTCTCTGCTGATGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGATGCGGTACAGGGGTGTTCGTCT
ATAACGACGTTGAAGCCTGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGATTGGCAGCAGCAGTCAAGCA
AGCCTGGGAAGATGGTATCTGCGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTGGAGATCAGTAGAAGGGGAG
CTCAACGCAATCCTGGAAGAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTC
CACAGAGATTGCCCGTGCCTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGCACTTCGTCAGAGCAGC
AAAGACAAATAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTC
TTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGAT
CCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGA
ATGACACATGGAGGCTGAAGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGAC
AGATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCT
ACAGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCA
CGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGATCGAGGAATGGTG
CTGCAGGGAGTGCACAATGCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGAATGGAGATAAGGCCCAGG
AAAGAACCAGAAAGCAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGT
GCTTGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCAATGGCA
GTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTTGATGGGTGCCACCTTCGC
GGAAATGAACACTGGAGGAGATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGACCAGCGTTGCTGGTATCTT
TCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTTTGCAAACTGCGATCTCCG
CCTTGGAAGGCGACCTGATGGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCA
CTGATAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGGAGAGCAGGC
CTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAACTTACCATTTGTCATGGC
CCTGGGACTAACCGCTGTGAGGCTGGTCGACCCCATCAACGTGGTGGGGCTGCTGTTGCTCACAAGGAGTGGGAAGCGG
AGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTCGCCAAGGCAGATATAGA
GATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGAGTGTGGACATGTACATTG

AAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCGATGTGGCGCTAGATG
AGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAGGTGGTCCTGATGACCATC
TGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACTGGAAAAAGGAGTGGTG
CTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAG
ACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACAAAAGGA
TCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGTCATACTGTGGTC
CATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGA
ACATCCAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAAC
TTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGT
TATGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGCCTTCGATGCTGAAGAAGA
AGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAAATAGTCCGTGAAGCCATA
AAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTTCC
AGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAATGTGCCATGCCACCTTCAC
TTCACGTCTACTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCACTTCACAGATCCCTCAAGT
ATAGCAGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCATGACCGCCACGCCACCAG
GAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCA
GGCTTTGATTGGGTGACGGATTATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAACGGCAATGAGATCGCAGC
TTGTCTGACAAAGGCTGGAAAACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGTTCCAGAAAACAAAACATC
AAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGACCGTGTCATAGATTCCAGG
AGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGTCACACATGCCAGCGCTGC
CCAGAGGAGGGGGCGCATAGGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGAC
TGACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGATGGCCTCATAGCCTCGCT
CTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACCTTTGTG
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SEQ ID NO: 3
KU497555.1 Zika virus isolate Brazil-ZKV2015, Brazil, complete genome
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SEQ ID NO: 4

KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome

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SEQ ID NO: 6

KU527068.1 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, complete genome

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SEQ ID NO: 7

KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome

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SEQ ID NO: 9
KU707826.1 Zika virus isolate SSABR1, Brazil, complete genome
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SEQ ID NO: 10
KU744693.1 Zika virus isolate VE_Ganxian, China, complete genome
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SEQ ID NO: 11

LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome

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SEQ ID NO: 12
AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome
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SEQ ID NO: 13
KJ776791.1, Zika virus strain H/PF/2013 polyprotein gene, complete cds
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TGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGA
AGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACA
CATGACAGAGAGATTCCAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAA
GCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTT
TTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATG
TGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAG
CCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTAC
CGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAAC
CATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCA
GCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGT
ATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC
CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGC
GTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACAC
AATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGT
CGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGA
ACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTA
CACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGG
CCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCC
CGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTG
GAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCA
CCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTA
TGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTG
ACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTT
GAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCA
GCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCG
AAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGA
AATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCAT
TGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGG
GCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAA
ACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAG
GAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGA
AAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATT
AGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGT
GGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGA
AACAAGGGGAATTTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGA
AGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGAGAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACA
AAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCAT
TGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGA
CATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGC
AACTCATTCGGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGAC
CAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATT
GATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAAACCCT
CAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCC
ATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGG
AGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCA
ATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAATGG
ATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACCACATGGAAGACAAGACCCCA

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GTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGA
CTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTC
AGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTA
TAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACC
CCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGAT
CAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAG

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13 or 72. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13 or 72.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 14
isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 15
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
RDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 16
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXXXXXXXNR
AEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 17

ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMXXXXXGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 18
ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 19
ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 20
isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 21
isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 22
isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVE
FKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVT

VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 23
MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 24
LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 25
isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 26
ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 27
AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 28

MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 29
MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 30
KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 31
isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 32
CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 33
Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.]

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 34
isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 35
KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 36
KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 37
isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 38
BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF

EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 39
MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 40
KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 41
Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 42
KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 43
isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 44

isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 45
PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 46
BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 47
H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 48
PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 49
KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 50
ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 51
103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 52
isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 53
KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 54
isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 55
isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 56
isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 57
isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 58
isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 59
isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNGTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 60
isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF

KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 61
ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 62
KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 63
isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 64
isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCPTQGEAYLD
KQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENR
AKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFK
DAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVE
VQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 65
isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAF

EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 66
KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 67
isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 68
ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E.
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCPTQGEPSL
DKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 69
MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 70
5′-(dIdC)$_{13}$-3′
dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC

SEQ ID NO: 71
KLK peptide
KLKLLLLLKLK

SEQ ID NO: 72
ZIKV Sequence H/PF/2013 as sequenced
CAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTT
TCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTG

AGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCT
AGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAAAAGAGGCTA
TGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGAAGGAGAAGAAGAGAC
GAGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAG
TGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTT
ATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAA
CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCACAAAAAAGGTGAAGC
ACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGTCGCAAACCTGGTTGGAAT
CAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCC
ATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGC
ATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAAC
ATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTACAACAACAGTCAGCAACAT
GGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCGGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAA
GCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTG
GACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCC
AGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGACACAGGACATG
AAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACCCTGGGGGGTTTTGGAAG
CCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATGAATAACAAGCACTGGTT
GGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAACTCCACACTGGAACAACA
AAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAG
TTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCTCTGGCCACTTGAAATG
TCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCC
GGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAG
ATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAA
CTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGGGAGAAGAAGATCACCC
ACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTGAAGCCACTGTGAGAGGTGCCAAGAGAATGGCAGTCTT
GGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCTCAACTCATTGGGCAAGGGCATCCATCAAATTTTTGGAG
CAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACAAATTCTCATTGGAACGTTGCTGATGTGGTTGGGTCTGA
ACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGGGGGAGTGTTGATCTTCTTATCCACAGCTGTCTCTGCTGA
TGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGATGCGGTACAGGGGTGTTCGTCTATAACGACGTTGAAGCC
TGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGATTGGCAGCAGCAGTCAAGCAAGCCTGGGAAGATGGTA
TCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTGGAGATCAGTAGAAGGGGAGCTCAACGCAATCCTGGAA
GAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGC
CTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGTACTTCGTCAGAGCAGCAAAGACAAATAACAGCTT
TGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTCTTGTGGAGGATCATGGGT
TCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAG
CTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGAATGACACATGGAGGCTGA
AGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGACAGATGGAATAGAAGAGAG
TGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCTACAGGACCCAAATGAAAG
GGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGG
AACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGATCGAGGAATGGTGCTGCAGGGAGTGCACAAT
GCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGAATGGAGATAAGGCCCAGGAAAGAACCAGAAAGTAAC
TTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGTGCTTGTGATTCTGCTCATG
GTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCGATGGCAGTGCTGGTAGCTATGATCC
TGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTTGATGGGTGCCACCTTCGCGGAAATGAACACTGGAGGA
GATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGACCAGCGTTGCTGGTATCTTTCATCTTCAGAGCTAATTGG
ACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTTTGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGATG
GTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCACTGATAACATCACCTTGGCA
ATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTT
TATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAACTTACCATTTGTCATGGCCCTGGGACTAACCGCTGTGA
GGCTGGTCGACCCCATCAACGTGGTGGGACTGCTGTTGCTCACAAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGT
ACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTCGCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCC
GCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGAGTGTGGACATGTACATTGAAAGAGCAGGTGACATCAC
ATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTG
GTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAGGTGGTCCTGATGACCATCTGTGGCATGAACCCAATAGC

CATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACTGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTC
CCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGT
TGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAA
GGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCT
GGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAA
TATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAACTTCAGGATCTCCAATCCTAGAC
AAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAG
GGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGCCTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTT
GCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAAATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGA
TCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCA
GTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAATGTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCA
GAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCACTTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTT
CAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCATGACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGA
CTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATC
ATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAACGGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAA
ACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGTTCCAGAAAACAAAACATCAAGAGTGGGACTTTGTCGTG
ACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGACCGTGTCATAGATTCCAGGAGATGCCTAAAGCCGGTCAT
ACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGTCACACATGCCAGCGCTGCCCAGAGGAGGGGGCGCATA
GGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGACTGACGAAGACCATGCACACT
GGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGATGGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACA
AAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACCTTTGTGGAACTCATGAAAAGAGGAG
ATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAATAACCTACACAGATAGAAGATGGTGCTTTGATGGCACGA
CCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTGTGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGA
GGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGAAGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGC
GGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACACATGACAGAGAGATTCCAGGAAGCCATTGACAACCTC
GCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAAGCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCA
TTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTTTTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATG
GGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATGTGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGT
CCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAGCCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGC
AATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTACCGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTG
ACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAACCATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTC
AGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCAGCCGTCCAACATGCAGTGACCACTTCATACAACAACTAC
TCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGTATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGG
AGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACCCCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCAC
TACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGCGTCGTCCCCAGAAGAGAACGGCAGCTGGCATCATGAAGA
ACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACACAATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACA
GGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGTCGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTG
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ATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTACACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGG
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GCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTGGAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATC
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AGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTATGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTC
TTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTGACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGC
ACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTTGAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCC
CATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCAGCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTC
CCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCGAAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGC
TCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGAAATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGG
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AACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGGGCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAG
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GAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATTAGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAG

TGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGTGGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGA
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GGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGAAGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGA
GAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACAAAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGA
GGAAGGATGTATGCAGATGACACTGCTGGCTGGGACACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCA
CCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCATTGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGT
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GACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGACCAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATG
GCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATTGATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGG
AAAAGTTAGGAAGGACACACAAGAGTGGAAACCCTCAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCAC
CACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCCATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGC
CCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGGAGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTC
CTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCAATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGG
AGAACTACCTGGTCAATCCATGGAAAGGGAGAATGGATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGA
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GCAGGATCATAGGTGATGAAGAAAAGTACATGGACTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACA
CCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTCAGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGT
GACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTATAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCT
GTGAGCCCCTCAGAGGACACTGAGTCAAAAAACCCCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCT
TCCCCACCCTTCAATCTGGGGCCTGAACTGGAGATCAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAGACCC
CCCGGAAAACGCAAAACAGCATATTGACGCTGGGAAAGACCAGAGACTCCATGAGTTTCCACCACGCTGGCCGCCAGGC
ACAGATCGCCGAATAGCGGCGGCCGGTGTGGGG

SEQ ID NO: 73

AHZ13508.1, Zika virus polyprotein from Polynesian outbreak (H/PF/2013)

MKNPKKKSGGFRIVNMLKRGVARVSPFGGLKRLPAGLLLGHGPIRMVLAILAFLRFTAIKPSLGLINRWGSVGKKEAMEIIKKFKK
DLAAMLRIINARKEKKRRGADTSVGIVGLLLTTAMAAEVTRRGSAYYMYLDRNDAGEAISFPTTLGMNKCYIQIMDLGHMCDA
TMSYECPMLDEGVEPDDVDCWCNTTSTWVVYGTCHHKKGEARRSRRAVTLPSHSTRKLQTRSQTWLESREYTKHLIRVENWI
FRNPGFALAAAAIAWLLGSSTSQKVIYLVMILLIAPAYSIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIEL
VTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTG
KSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKH
WLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLK
CRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKM
MLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLF
GGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSADVGCSVDFSKKETRCGTGVFVYNDVEAWRDRYKYHP
DSPRRLAAAVKQAWEDGICGISSVSRMENIMWRSVEGELNAILEENGVQLTVVVGSVKNPMWRGPQRLPVPVNELPHGWK
AWGKSYFVRAAKTNNSFVVDGDTLKECPLKHRAWNSFLVEDHGFGVFHTSVWLKVREDYSLECDPAVIGTAVKGKEAVHSDL
GYWIESEKNDTWRLKRAHLIEMKTCEWPKSHTLWTDGIEESDLIIPKSLAGPLSHHNTREGYRTQMKGPWHSEELEIRFEECPG
TKVHVEETCGTRGPSLRSTTASGRVIEEWCCRECTMPPLSFRAKDGCWYGMEIRPRKEPESNLVRSMVTAGSTDHMDHFSLG
VLVILLMVQEGLKKRMTTKIIISTSMAVLVAMILGGFSMSDLAKLAILMGATFAEMNTGGDVAHLALIAAFKVRPALLVSFIFRA
NWTPRESMLLALASCLLQTAISALEGDLMVLINGFALAWLAIRAMVVPRTDNITLAILAALTPLARGTLLVAWRAGLATCGGFM
LLSLKGKGSVKKNLPFVMALGLTAVRLVDPINVVGLLLLTRSGKRSWPPSEVLTAVGLICALAGGFAKADIEMAGPMAAVGLLIV
SYVVSGKSVDMYIERAGDITWEKDAEVTGNSPRLDVALDESGDFSLVEDDGPPMREIILKVVLMTICGMNPIAIPFAAGAWYVY
VKTGKRSGALWDVPAPKEVKKGETTDGVYRVMTRRLLGSTQVGVGVMQEGVFHTMWHVTKGSALRSGEGRLDPYWGDVK
QDLVSYCGPWKLDAAWDGHSEVQLLAVPPGERARNIQTLPGIFKTKDGDIGAVALDYPAGTSGSPILDKCGRVIGLYGNGVVIK
NGSYVSAITQGRREEETPVECFEPSMLKKKQLTVLDLHPGAGKTRRVLPEIVREAIKTRLRTVILAPTRVVAAEMEEALRGLPVRY
MTTAVNVTHSGTEIVDLMCHATFTSRLLQPIRVPNYNLYIMDEAHFTDPSSIAARGYISTRVEMGEAAAIFMTATPPGTRDAFP
DSNSPIMDTEVEVPERAWSSGFDWVTDHSGKTVWFVPSVRNGNEIAACLTKAGKRVIQLSRKTFETEFQKTKHQEWDFVVTT
DISEMGANFKADRVIDSRRCLKPVILDGERVILAGPMPVTHASAAQRRGRIGRNPNKPGDEYLYGGGCAETDEDHAHWLEAR
MLLDNIYLQDGLIASLYRPEADKVAAIEGEFKLRTEQRKTFVELMKRGDLPVWLAYQVASAGITYTDRRWCFDGTTNNTIMEDS
VPAEVWTRHGEKRVLKPRWMDARVCSDHAALKSFKEFAAGKRGAAFGVMEALGTLPGHMTERFQEAIDNLAVLMRAETGS
RPYKAAAAQLPETLETIMLLGLLGTVSLGIFFVLMRNKGIGKMGFGMVTLGASAWLMWLSEIEPARIACVLIVVFLLLVVLIPEPE
KQRSPQDNQMAIIIMVAVGLLGLITANELGWLERTKSDLSHLMGRREEGATIGFSMDIDLRPASAWAIYAALTTFITPAVQHAV

TTSYNNYSLMAMATQAGVLFGMGKGMPFYAWDFGVPLLMIGCYSQLTPLTLIVAIILLVAHYMYLIPGLQAAAARAAQKRTAA
GIMKNPVVDGIVVTDIDTMTIDPQVEKKMGQVLLIAVAVSSAILSRTAWGWGEAGALITAATSTLWEGSPNKYWNSSTATSLC
NIFRGSYLAGASLIYTVTRNAGLVKRRGGGTGETLGEKWKARLNQMSALEFYSYKKSGITEVCREEARRALKDGVATGGHAVSR
GSAKLRWLVERGYLQPYGKVIDLGCGRGGWSYYAATIRKVQEVKGYTKGGPGHEEPMLVQSYGWNIVRLKSGVDVFHMAAE
PCDTLLCDIGESSSSPEVEEARTLRVLSMVGDWLEKRPGAFCIKVLCPYTSTMMETLERLQRRYGGGLVRVPLSRNSTHEMYWV
SGAKSNTIKSVSTTSQLLLGRMDGPRRPVKYEEDVNLGSGTRAVVSCAEAPNMKIIGNRIERIRSEHAETWFFDENHPYRTWAY
HGSYEAPTQGSASSLINGVVRLLSKPWDVVTGVTGIAMTDTTPYGQQRVFKEKVDTRVPDPQEGTRQVMSMVSSWLWKELG
KHKRPRVCTKEEFINKVRSNAALGAIFEEEKEWKTAVEAVNDPRFWALVDKEREHHLRGECQSCVYNMMGKREKKQGEFGKA
KGSRAIWYMWLGARFLEFEALGFLNEDHWMGRENSGGGVEGLGLQRLGYVLEEMSRIPGGRMYADDTAGWDTRISRFDLE
NEALITNQMEKGHRALALAIIKYTYQNKVVKVLRPAEKGKTVMDIISRQDQRGSGQVVTYALNTFTNLVVQLIRNMEAEEVLEM
QDLWLLRRSEKVTNWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRFLNDMGKVRKDTQEWKPSTGWDNWEEVPFC
SHHFNKLHLKDGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQMWQLLYFHRRDLRLMANAICSSVPVDWVPTG
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SEQ ID NO: 74
9320_Zika_PF_1F
ttaggatccGTTGTTGATCTGTGTGAAT

SEQ ID NO: 75
9321_Zika_PF_1R
taactcgagCGTACACAACCCAAGTT

SEQ ID NO: 76
9322_Zika_PF_2F
ttaggatccTCACTAGACGTGGGAGTG

SEQ ID NO: 77
9323_Zika_PF_2R
taactcgagAAGCCATGTCYGATATTGAT

SEQ ID NO: 78
9324_Zika_PF_3F
ttaggatccGCATACAGCATCAGGTG

SEQ ID NO: 79
9325_Zika_PF_3R
taactcgagTGTGGAGTTCCGGTGTCT

SEQ ID NO: 80
9326_Zika_PF_4F
ttaggatccGAATAGAGCGAARGTTGAGATA

SEQ ID NO: 81
9327_Zika_PF_4R
taactcgAGTGGTGGGTGATCTTCTTCT

SEQ ID NO: 82
9328_Zika_PF_5F
ttaggatcCAGTCACAGTGGAGGTACAGTAC

SEQ ID NO: 83
9329_Zika_PF_5R
taactcgagCRCAGATACCATCTTCCC

SEQ ID NO: 84
9330_Zika_PF_6F
ttaggatCCCTTATGTGCTTGGCCTTAG

SEQ ID NO: 85
9331_Zika_PF_6R
taactcgagTCTTCAGCCTCCATGTG

SEQ ID NO: 86
9332_Zika_PF_7F
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SEQ ID NO: 87
9333_Zika_PF_7R
taactcgagTCATTCTCTTCTTCAGCCCTT

SEQ ID NO: 88
9334_Zika_PF_8F
ttaggatccAAGGGTGATCGAGGAAT

SEQ ID NO: 89
9335_Zika_PF_8R
taactcgagTTCCCTTCAGAGAGAGGAGC

SEQ ID NO: 90
9336_Zika_PF_9F
ttaggatccTCTTTTGCAAACTGCGATC

SEQ ID NO: 91
9337_Zika_PF_9R
taactcgagTCCAGCTGCAAAGGGTAT

SEQ ID NO: 92
9338_Zika_PF_10F
ttaggatccGTGTGGACATGTACATTGA

SEQ ID NO: 93
9339_Zika_PF_10R
taactcgagCCCATTGCCATAAAGTC

SEQ ID NO: 94
9340_Zika_PF_11F
ttaggatccTCATACTGTGGTCCATGGA

SEQ ID NO: 95
9341_Zika_PF_11R
taactcgagGCCCATCTCAACCCTTG

SEQ ID NO: 96
9342_Zika_PF_12F
ttaggatccTAGAGGGCTTCCAGTGC

SEQ ID NO: 97
9343_Zika_PF_12R
taactcgAGATACTCATCTCCAGGTTTGTTG

SEQ ID NO: 98
9344_Zika_PF_13F
ttaggatccGAAAACAAAACATCAAGAGTG

SEQ ID NO: 99
9345_Zika_PF_13R
taactcgagGAATCTCTCTGTCATGTGTCCT

SEQ ID NO: 100
9346_Zika_PF_14F
ttaggatccTTGATGGCACGACCAAC

SEQ ID NO: 101
9347_Zika_PF_14R
ttaggatccGTTGTTGATCTGTGTGAAT

SEQ ID NO: 102
9348_Zika_PF_15F
taactcgagCAGGTCAATGTCCATTG

SEQ ID NO: 103
9349_Zika_PF_15R
ttaggatccTGTTGTGTTCCTATTGCTGGT

SEQ ID NO: 104
9350_Zika_PF_16F
taactcgaGTGATCAGRGCCCCAGC

SEQ ID NO: 105
9351_Zika_PF_16R
ttaggatccTGCTGCCCAGAAGAGAA

SEQ ID NO: 106
9352_Zika_PF_17F
taactcgaGCACCAACAYGGGTTCTT

SEQ ID NO: 107
9353_Zika_PF_17R
ttaggatcCTCAAGGACGGTGTGGC

SEQ ID NO: 108
9354_Zika_PF_18F
taactcgagCAATGATCTTCATGTTGGG

SEQ ID NO: 109
9355_Zika_PF_18R
ttaggatccTATGGGGGAGGACTGGT

SEQ ID NO: 110
9356_Zika_PF_19F
taactcGAGCCCAGAACCTTGGATC

SEQ ID NO: 111
9357_Zika_PF_19R

ttaggatcCAGACCCCCAAGAAGGC

SEQ ID NO: 112
9358_Zika_PF_20F
taactcgagCCCCTTTGGTCTTGTCT

SEQ ID NO: 113
9359_Zika_PF_20R
ttaggatccAGGAAGGATGTATGCAGATG

SEQ ID NO: 114
9360_Zika_PF_21F
taactcgagACATTTGCGCATATGATTTTG

SEQ ID NO: 115
9361_Zika_PF_21R
ttaggatccAGGAAGGACACACAAGAGT

SEQ ID NO: 116
9362_Zika_PF_22F
taactcgagACAGGCTGCACAGCTTT

SEQ ID NO: 117
9363_Zika_PF_22R
ttaggatccTCTCTCATAGGGCACAGAC

In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NOs: 14-69 or 72. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69 or 72.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. MoI. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Dr., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977 and Altschul et al., J. MoI. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were adapted to ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 9A and B). Briefly, the unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Zika Virus. As shown in Figure 10, non-infectious virus particle aggregates, HCP and other LMW impurities were removed by PS precipitation as shown by removal of aggregate shoulder in SEC-HPLC and no loss of infectious virus titer by PS treatment. Further optimization of the Zika purification protocol is provided below.

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):
- 5% CO_2, 35°C, MEM+2mM L-Glutamine + 10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01

- Virus Production without serum
- 5% CO_2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (accession number KJ776791.1, also SEQ ID NO: 13 herein) from French Polynesia. A sample was obtained from the European Virus Archive (EVAg; Ref-SKU: 001v-EVA1545). Based on this material, a research master seed bank (rMSB) was prepared on Vero cells as the cell substrate and the genomic sequence was checked by sequencing. Because the genomic sequence at the 5´and 3´flanking sequences of Zika virus strain H/PF/2013 was unknown, primers for sequencing were designed in those regions based on other Zika virus strains whereas the internal primers were designed from the published sequence (SEQ ID NOs: 74 to 117, see also Table A). The sequence obtained from the rMSB by use of these primers is provided by SEQ ID NO: 72. There was 100% overlap of the sequence with the published sequence of Zika Virus Strain H/PF/2013 (SEQ ID NO: 13). However, we sequenced additional regions 5´ (an additional 40 bp) and 3' (an additional 160 bp) represented in SEQ ID NO: 72. In a preferred embodiment, the Zika virus of the invention comprises SEQ ID NO: 72. The genomic RNA is somewhat longer than the sequence according to SEQ ID NO: 72 (perhaps an additional 200 bp). Additionally, a Zika virus adapted to a host cell such as e.g. Vero cells may be expected to contain one or more mutations. For these reasons, the Zika virus of the current invention comprises the sequence of SEQ ID NO: 72 or, preferably, a sequence with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 72. Furthermore, because the viral genome is likely to contain even further flanking regions to SEQ ID NO: 72; in one embodiment, the Zika virus of the invention contains the sequence of SEQ ID NO: 72 and optionally further comprises extensions at the 5' and/or 3' ends of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120 or at least 130 nucleotides. In a preferred embodiment, the Zika virus comprises at least the coding sequence for the entire

polyprotein of Zika Virus Strain H/PF/2013 of the invention i.e. the amino acid sequence of SEQ ID NO: 73 or a polyprotein with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 73. Furthermore, the Zika virus comprises at least the coding sequence for the E-protein of Zika Virus Strain H/PF/2013 of the invention SEQ ID NO: 47 or an E-protein thereof with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 47.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in a standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and to reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles were infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200 mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significantly higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with the day 3 harvest, the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest.

Table 3: The calculated titers per plaque assay are summarized in the list below.

	Log 10 PFU/mL
sample day2	7.02
harvest day 3	6.66
harvest day 5	6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 15. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22°C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 11 for SEC-HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 20 for SEC-HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 21). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

Samples of individual 30x concentrated harvests days 2, 3, 5 and 7 were analysed before (Figure 11) and after PS (Figure 12) treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for

determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 4. The virus peak recovery by SEC-HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 4: ZikaV recovery by SEC-HPLC and plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x + PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x + PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles. Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus particles from residual contaminants as demonstrated for ChikV (Fig. 15D). The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 5.

Table 5: Individual layers/volumes for a centrifugation in bottle.

Solution	**Volume (mL)**
PS treated harvest in 10% sucrose (L)	40
15% sucrose (J)	15
35% sucrose (I)	15
50% sucrose (H)	20
Total volume	90

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 14. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled rotor. (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers.) The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no brake/deceleration activated. In case a different centrifuge system with a different rotor is used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle (see Figure 14).

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL)

and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 14. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51 mL in a PETG container. A volume of 1% formaldehyde (10,000 ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200 ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25°C) for a minimum of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criterion for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution was monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to

unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152 µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. Briefly, a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 21.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gently mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 6).

Table 6: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	JEV	Zika
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2 µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565.

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 16. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 17) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6 nm and ~49 nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 18).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Table 7: Active ZikaV pfus were quantified by plaque assay throughout the process.

Sample	Pfu/mL
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$
Harvest day 5 (filtered)	$1.5x10^8$
Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD

Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2 mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly, the Benzonase treated samples did not yield any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell protein was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well was seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5% CO_2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicate. The target viruses (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were diluted to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were

incubated at 35°C with 5% CO_2 for 1 hour. The cell culture medium was aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plates were rocked back and forth 5 times before incubating for 2 hours at 35°C with 5% CO_2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5% CO_2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 19 and 20, respectively). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Another neutralization assay was performed using the microneutralization assay as described by Larocca, et al. (2016, Nature doi:10.1038/nature18952). It was found that the inactivated Zika virus of the current invention had an MN50 (microneutralization) titer of 90 at 1 µg of inactivated purified virus.

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by

administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on Vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV vaccine. The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

The in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-

protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, supra.). Inactivated viruses are among the safest vaccines and especially preferred for deliver to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

A4. The vaccine of any one of A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of A1-A9, further comprising an adjuvant.

A11. The vaccine of A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

A15. The vaccine of any one of A1-A14, further comprising one or more pharmaceutically acceptable excipient.

A16. The vaccine of any one of A1-A15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 µg/mL, especially below 100 ng/mL.

A17. The vaccine of A16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

B1. A kit comprising a Zika virus vaccine of any one of A1-A15.

B2. The kit of B1, further comprising a second vaccine.

B3. The kit of B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of A1-A15 to a subject in need thereof.

C2. The method of C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of A1-A15 for the treatment and/or prevention of a Zika virus infection.

E2. The use of E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment or prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of A1-A15.

F2. The pharmaceutical composition of F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

G1. Use of an optimized sucrose gradient centrifugation for removal of protamine sulphate from purified infectious Zika virus particles.

G2. The use according to G1, wherein said optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three further layers of sucrose solutions with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G3. A process of purification of infectious Zika virus particles, comprising the steps of:

a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

G4. The process of G3, wherein said optimized sucrose density gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G5. The process of any one of G3 to G4, additionally comprising a further purification step of: (d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from

entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

G6. The process of any of G3 to G5, wherein the residual host cell DNA content of the Zika virus preparation (c) is less than 10 ng/mL and the residual host cell protein content of the final virus preparation (c) is less than 100 ng/mL.

G7. The process of any of G3 to G6, wherein said crude harvest (a) comprising Zika virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

G8. The process of G7, wherein the one or more pre-purification step(s) comprises

a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

b) digestion of host cell genomic DNA by enzymatic treatment; and/or

c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

G9. The process of any one of G3 to G8, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

G10. The process of any one of G3 to G9, wherein the enrichment of infectious Zika virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

G11. The process of any one of G7 to G10, wherein the one or more pre-purification step(s) prior to step (b) of any of G8 to G11 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

G12. The process of any one of G3 to G11, wherein the residual impurity of the Zika virus preparation (c) is less than 10%.

G13. The process of any one of G3 to G12, wherein the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a

PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

G14. The process of G13, wherein said cell line is a Vero cell line.

G15. The process of any one of G3 to G14, wherein said infectious Zika virus particle is an infectious virus particle that is a live virus, a live attenuated virus, a chimeric virus, a modified live virus, or a recombinant live virus.

G16. The process of any one of G3 to G15, wherein said Zika virus is preferably a strain of the Asian lineage.

G17. The process of any one of G3 to G16, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

G18. Use of the process according to any one of G3 to G17 for manufacturing a composition for immunization against a virus infection.

G19. The use according to G18, wherein said virus infection is an infection caused by a Zika virus.

Q1. A process of purification of infectious Zika virus particles, comprising the steps of:
(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;
(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);
(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

Q2. The process of Q2, wherein the virus particles are from Zika virus.

Q3. The process of Q1 or Q2, additionally comprising the step of:

(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

Q4. The process of any of Q1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

Q5. The process of any of Q1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

Q6. The process of Q5, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

Q7. The process of any one of Q1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

Q8. The process of any one of Q1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

Q9. The process of any one of Q5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of Q5 to 8 is performed using a filter having a pore size equal to or less than 1 µm, preferably 0.2 µm.

Q10. The process of any one of Q1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

Q11. The process of any one of Q1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

Q12. The process of Q11, wherein said cell line is a Vero cell line.

Q13. The process of any one of Q1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

Q14. The process of any one of Q1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

Q15. The process of any one of Q1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

Q16. Use of the process according to any one of Q1 to 15 for manufacturing a composition for immunization against a virus infection.

Q17. The use according to Q16, wherein the composition for immunization against a virus infection is an infection caused by Zika virus.

Q18. A composition comprising the virus particles obtainable or obtained by the process of any one of Q1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

Q19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

Q20. The Zika virus vaccine of Q19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

Q21. The vaccine of Q19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

Q22. The vaccine of any one of Q19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69 or 72, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 or 72 and able to pack a virulent Zika virus.

Q23. The vaccine of any one of Q19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

Q24. The vaccine of Q23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

Q25. The vaccine of Q24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

Q26. The vaccine of any one of Q19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

Q27. The vaccine of Q26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

Q28. The vaccine of Q27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

Q29. The vaccine of Q28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

Q30. The vaccine of any one of Q27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

Q31. The vaccine of any one of Q19 to 30, further comprising an adjuvant.

Q32. The vaccine of Q31, wherein the adjuvant is an aluminum salt adjuvant.

Q33. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

Q34. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

Q35. The vaccine of any one of Q19 to 34, further comprising one or more pharmaceutically acceptable excipient.

R1. Use of protamine, preferably a protamine salt, to separate infectious and non-infectious Zika virus particles, host cell proteins and/or undefined low molecular weight materials.

R2. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b), wherein the enrichment of infectious virus particles in the virus preparation (b) relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R3. The use of R1 or the process of R2, wherein the virus particles are from Zika virus.

R4. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by one or more size exclusion methods such as (i) a sucrose density gradient centrifugation, (ii) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles, and/or (iii) size exclusion chromatography to obtain a virus preparation (c) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (c) is less than 100 ng/mL and the residual host cell protein and the residual aggregates of infectious virus particles of the final virus preparation (c) is less than 1 μg/mL.

R5. The process of R4, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

R6. The process of any of R2 to 5, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

R7. The process of R6, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

R8. The process of any one of R2 to 7, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.

R9. The process of any one of R2 to 8, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R10. The process of any one of R6 to 9, wherein the one or more pre-purification step(s) prior to step (b) of any of R6 to 9 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

R11. The process of any one of R2 to 10, wherein the residual impurity of the virus preparation (c) is less than 10%.

R12. The process of any one of R2 to 11, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

R13. The process of R12, wherein said cell line is a Vero cell line.

R14. The process of any one of R2 to 13, wherein the Zika virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

R15. The process of any one of R2 to 14, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

R16. The process of any one of R2 to 15, wherein said process resulting in final virus preparation (c) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

R17. Use of the process according to any one of R1 to 16 for manufacturing a composition for immunization against a virus infection.

R18. The use according to R17, wherein the composition for immunization against a virus infection is an infection caused by a Zika virus.

R19. A composition comprising the virus particles obtainable or obtained by the process of any one of R2 to 16 for treating and/or preventing an infection.

ZIKA VIRUS VACCINE

RELATED APPLICATION

This Application is a national stage filing under 35 U.S.C. § 371 of International Patent Application Serial No. PCT/EP2016/082664, filed December 23, 2016, the contents of which is incorporated herein by reference in its entirety.

FIELD OF INVENTION

The disclosure relates to methods for the purification of Zika viruses for use in vaccines and in particular relates to an improved sucrose gradient process step allowing the separation of impurities such as protamine sulphate. The disclosure also relates to Zika virus vaccines and compositions and methods for producing said vaccines and administering the vaccines to subjects for the generation of an anti-Zika virus immune response.

BACKGROUND OF THE INVENTION

Adverse responses to protamine sulfate have been known for many years. Previous exposure to protamine can induce a humoral immune response and predispose susceptible individuals to the development of untoward reactions from the subsequent use of this drug. Patients exposed to protamine through the use of protamine-containing insulin or during heparin neutralization may experience life-threatening reactions and fatal anaphylaxis upon receiving large doses of protamine intravenously. Severe reactions to intravenous protamine can occur in the absence of local or systemic allergic reactions to subcutaneous injection of protamine-containing insulin. Although there is no clear evidence for hypersensitivity reactions of protamine sulphate linked to vaccination, vaccines containing protamine impurities have a precaution and contraindication warning in their labels stating that a serious allergic reaction after a previous dose of such a protamine containing vaccine (e.g. IXIARO®, see CDC site www.cdc.gov/japaneseencephalitis/vaccine/) is a contraindication to further doses. Thus elimination of said impurity is a medical request for an improved safety profile. On the other hand protamine sulphate is an excellent tool (and often better than other tools such as benzonase) to purify crude harvests of viruses grown on cell substrates.

In 2007, Zika virus was detected for the first time outside of the endemic regions of Asia and Africa since its discovery in a Rhesus monkey in Uganda in 1947. Since then, the virus has caused a large

epidemic in French Polynesia, spreading through islands in the Pacific and into South and Central America by 2015 (WHO "Zika Situation Report" February 5, 2016). Evidence suggests that in addition to being transmitted by *Aedes* species mosquitos, other vectors may exist, and the virus may be transmitted by blood transfusion, transplacentally, and through sexual transmission (WHO Zika Virus Fact Sheet, Feb. 2016). Though the symptoms of Zika virus infection include mild fever, rash, and conjunctivitis, there is a likely correlation between infection and neurological disorders, including Guillain-Barré syndrome and microcephaly in fetuses/neonates subsequent to infection during pregnancy. There is currently no specific treatment or vaccine for Zika virus and the only preventative measures involve control of the mosquito vector. Zika virus presents a substantial public health threat due to the wide circulation of the *Aedes* mosquito, multiple routes of transmission, and potentially severe neurological effects of infection.

A preventative vaccine against Zika virus is a pressing medical need in endemic areas and in geographical areas where the vector is spreading. Furthermore, as Zika infection has dire consequences on embryonic and fetal development, a safe and effective vaccine for women of child-bearing potential or pregnant women is needed. Vaccines administered during pregnancy must be very safe for both the mother and the developing fetus. While live attenuated viral vaccines are highly effective, they are often not considered safe enough for administration to pregnant women. In this regard, inactivated viral vaccines, which lack the ability to propagate in the vaccinated subject, are considered much safer. Development of an inactivated Zika virus vaccine for administration to at-risk patients would fill this need.

SUMMARY OF THE INVENTION

During the course of virus purification, it was observed that addition of protamine sulfate to a virus harvest produced on a cell substrate removed not only contaminating DNA derived from host cells, as expected, but surprisingly also virtually eliminated immature and otherwise non-infectious virus particles from the preparation. This finding provided a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation. In addition, it was surprisingly found that said protamine sulfate can be very efficiently separated from the virus fraction allowing for a safer vaccine produced at high yields.

Disclosed herein are virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said Zika virus vaccines for the prevention of Zika virus infection and/or for the

production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to Zika virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated Zika virus particle, wherein the Zika virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Detail experimental examples to the above are provided for Zika virus.

The herein disclosed in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was unexpectedly higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, Nature doi:10.1038/nature18952.). Inactivated viruses are among the safest vaccines and especially preferred for delivery to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing, alignments were performed with the multi alignment package Jalview (Waterhouse et al., 2009, Bioinformatics 25 (9) 1189-1191). In the drawings:

Figure 1: Average distance tree (by % identity, nt), complete genomes.

Figure 2: Neighbor joining tree (by % identity, nt), complete genomes.

Figure 3: Pairwise alignment-Jalview (% identity, nt), complete genomes.

Figure 4: Average distance tree (by % identity, aa), E-protein.

Figure 5: Neighbor joining tree (by % identity. aa), E-protein.

Figure 6: Pairwise alignment-Jalview (% identity, aa), E-protein.

Figures 7A-7C: Alignment (shading: % identity, aa), E-protein.

Figure 8: An example of virus particle maturation in the host cell. As observed in flaviviruses, full maturation of the particles requires proteolytic cleavage of the precursor membrane glycoprotein (prM) by the host protease furin. Not all prM molecules are cleaved, resulting in the release of mature, mosaic or immature-like conformations from the cells. Mosaic and immature forms are generally not infectious—only mature virions are infective and have hemagglutinin (HA) / TCID50 activity. (Figure adapted from Plevka, et al., Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centres, EMBO reports (2011) 12, 602–606).

Figure 9: An exemplary downstream virus purification process from the crude harvest to formulation of the drug substance (vaccine), a preferred embodiment of the process of the invention (**A**). A flow-chart of an exemplary virus inactivation process is shown in (**B**).

Figure 10: PS treatment results in selective removal of Zika virus aggregates and Vero HCP and LMW impurities (SEC-HPLC of 30x concentrated Zika Virus harvest day 5).

Figure 11: SEC-HPLC of individual 30x concentrated Zika harvest prior to PS treatment at different time points.

Figure 12: SEC-HPLC of individual 30x concentrated Zika harvest post PS treatment at different time points. The smaller graph indicates the observed cytopathic effect (CPE) over time.

Figure 13: Preparation of the sucrose gradient.

Figure 14: Representative SDS-PAGE from the sucrose gradient harvest of a Zika purification is shown.

Figure 15: Comparison of JEV and ZikaV harvest schedules/yields.

Figure 16: SEC-HPLC elution profile of ZikaV NIV. Data were processed on Dionex Ultimate 3000 / Superose 6 Increase column. Both panels are from the same chromatogram. The upper graph is the complete elution profile; the lower graph is an enlargement of the ZIKAV elution peak.

Figure 17: SEC-MALLS analysis of inactivated ZikaV.

Figure 18: Cumulative particle size distribution of Zika NIV.

Figure 19: Graphical representation of the neutralization of the Zika virus H/PF/2013 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 20: Graphical representation of the neutralization of the Zika virus MR766 with pooled mouse sera. The number of plaques without serum was set to 100%. The EC50 was calculated using the 3-parameter method.

Figure 21: Correlation between JEV antigen content in neutralized inactivated virus (NIV) analysed by ELISA and SEC-HPLC (Dionex Ultimate 3000, Superose 6 column).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering said virus vaccines for the prevention of virus infection and/or for the production of an anti-virus immune response in subjects, for example subjects at risk of being exposed to virus. In particular, the invention is directed to a virus vaccine comprising an optimally inactivated virus particle, wherein the virus particle in an appropriate dose is able to seroconvert a subject that is administered the virus vaccine with at least a 70% probability, preferably an 80% probability. Another advantage of the invention is that related methods of producing said vaccines and

compositions are very efficient and provide pure compositions largely devoid of impurities, in particular protamine sulphate, allowing for high volume production of vaccines. Examples to the above are provided for Zika virus.

Disclosed herein are downstream processes for purifying Zika virus particles from a crude preparation. The downstream process can be applied to either a virus which has not adapted for propagation on a particular cell substrate or for a partially / fully cell substrate adapted Zika virus particle.

Aspects of the invention provide processes for the purification of infectious Zika virus particles comprising the steps of (a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate; (b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising a protamine salt, preferably a protamine sulphate, to obtain a virus preparation (b); and further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation to obtain a virus preparation (c) comprising the infectious virus particles.

In some embodiments, the concentration of protamine sulphate in step (b) is about 1 to 10 mg/ml, more preferably about 1 to 5 mg/ml, more preferably about 1 to 2 mg/ml. In one embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/mL. In one embodiment, the concentration of protamine sulphate is 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml. In a preferred embodiment, the concentration of protamine sulphate in step (b) is about 2 mg/ml.

In some embodiments, the residual host cell DNA of the virus preparation (c) is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL. In a preferred embodiment, the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the residual host cell protein of the virus preparation (c) is less than 100 ng/mL. In some embodiments, the residual non-infectious virus particles of the final virus preparation (c) is less than 10 µg/mL, especially less than 9, 8, 7, 6, 5, 4, 3 or 2 µg/mL, preferably less than 1 µg/mL. In a preferred embodiment, the content of residual non-infectious virus particles of the virus preparation (c) is less than 100 ng/mL.

In some embodiments, the residual protamine is less than 1 µg/mL, especially less than 900, 800, 700, 600, 500, 400, 300 or 200 ng/mL, preferably less than 100 ng/mL, more preferably is below the detection limit of HPLC, in particular below the detection limit in the final drug substance. In some embodiments, the PS content is tested by HPLC or size exclusion chromatography (SEC). For example, HPLC is validated for PS determination in JEV sucrose gradient pool samples as a routine release assay and is very sensitive (i.e., LOQ 3 µg/mL; LOD 1 µg/mL). In the current invention, PS content in in Zika virus DS samples was <LOD. In one embodiment, the HPLC assessment of PS content can be performed on a Superdex Peptide 10/300GL column (GE: 17-5176-01) using 30% Acetonitrile, 0,1% Trifluoroacetic acid as solvent with a flow rate of 0,6 ml/min at 25°C and detection at 214 nm. A more sensitive method of measurement for residual protamine in a purified virus preparation is mass spectrometry (MS). In some embodiments, the residual PS levels in a Zika virus preparation are tested by MS or other such highly sensitive method, e.g. nuclear magnetic resonance (NMR). With this method, residual PS, as well as fragments and/or break-down products of PS, can be detected at trace amounts, such as levels as low as, for example, 10^6, 10^7 or 10^8 molecules per typical sample load. In some embodiments, the PS levels are tested in the sucrose gradient pool. In some embodiments, the PS levels are tested in the drug product. In some embodiments, the PS levels are tested in the drug substance.

In some embodiments, the crude harvest (a) comprising the virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b). In some embodiments, the one or more pre-purification step(s) comprises digesting host cell genomic DNA in the crude harvest (a) comprising the virus particles and impurities by enzymatic treatment. In some embodiments, the one or more pre-purification step(s) comprises filtration, ultrafiltration, concentration, buffer exchange and/or diafiltration. In some embodiments, the one or more pre-purification steps is filtration using a filter having a pore size equal to or less than 1 µm. In some embodiments, the filter has a pore size equal to or less than 0.2 µm. In a preferred embodiment, the filter has a pore size of 0.2 µm. In some embodiments, the concentration and/or ultra/diafiltration and/or buffer exchange is performed by tangential flow filtration (TFF). In some embodiments, ultra/diafiltration of the crude harvest (a) comprising the virus particles and impurities is performed using a hollow fiber membrane having a cut-off of equal to or less than 300 kDa. In a preferred embodiment, the hollow fiber membrane has a cut-off of about 100 kDa.

The process according to the current invention may also comprise the use of a sucrose gradient, preferably an optimized sucrose gradient. The sucrose gradient is preferably optimized for the removal of protamine sulfate, also for the removal of immature viral particles or other viral particles which are non-infectious or host cell proteins or nucleic acids (DNA, RNA, mRNA, etc) or other host

cell debris. In the current invention the optimized sucrose gradient comprises at least two, at least three, at least four layers of sucrose solutions with different densities. In one embodiment, the virus preparation to be purified is provided in a sucrose solution which has a density of about 8%, about 9%, about 10%, about 11%, about 12% sucrose (w/w), preferably about 10%. In one embodiment, one sucrose solution in the gradient has a density of about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55% sucrose (w/w), preferably about 50%. In one embodiment, one sucrose solution in the gradient has a density of about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40% sucrose (w/w), preferably about 35%. In one embodiment, one sucrose solution in the gradient has a density of about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20% sucrose (w/w), preferably about 15% sucrose. In a preferred embodiment, the sucrose gradient comprises three layers of sucrose solutions of about 50%, about 35% and about 15% (w/w) sucrose and the virus composition to be purified is contained in about 10% (w/w) sucrose. Because the invention provided for means to not only test for host cell DNA but also immature viral particles, the skilled person in the art is able to more precisely optimize the sucrose gradient for most efficient purification and include additional tools such as PRNT assay to monitor purification success.

In some embodiments, the virus particle is a live virus, a chimeric virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In a further step, the virus particles of the invention may be optionally inactivated. In some embodiments, the virus particle is an attenuated form of the virus particle. For example, the virus may have reduced infectivity, virulence, and/or replication in a host, as compared to a wild-type virus. In some embodiments, the virus is a mutated or modified virus, for example the nucleic acid of the virus may contain at least one mutation relative to the wild-type virus. In some embodiments, the virus is a recombinant live virus, meaning a virus that is generated recombinantly and may contain nucleic acid from different sources.

In some embodiments, the Zika virus particle is a live virus, an attenuated live virus, a modified live virus, or a recombinant live virus. In preferred embodiments, the Zika virus is a Zika virus from the Asian lineage.

In some embodiments, the relative reduction of impurity of the final virus preparation relative to the liquid medium (a) comprising the virus particles and impurities is in a range from 60 to 95%. In some embodiments, the residual impurity of the final virus preparation is less than 1%.

In some embodiments, the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line. In some embodiments, said cell line is a duck cell line. In some embodiments, said cell line is a diploid avian cell line. In some embodiments, said cell line is an EB66 cell line. In a preferred embodiment, said cell line is a Vero cell line.

Aspects of the invention provide a use of any of the processes described herein for manufacturing a composition for immunization against a viral infection. In a preferred embodiment, the composition or vaccine is directed against Zika virus such as e.g. a Zika virus of the Asian lineage.

Other aspects provide compositions comprising the Zika virus particles obtainable by any of the processes described herein for treating and/or preventing (i.e. protecting from) a viral infection. In a preferred embodiment, the viral infection is caused by Zika virus such as e.g. a Zika virus of the Asian lineage.

Furthermore, disclosed herein are Zika virus vaccines and compositions comprising an inactivated Zika virus, and related methods of producing said vaccines and compositions. Also provided are methods of administering the Zika virus vaccines for the prevention of Zika virus infection and/or for the production of an anti-Zika virus immune response in subjects, for example subjects at risk of being exposed to Zika virus.

Zika virus is a flavivirus closely related to Dengue virus and is similarly transmitted by the *Aedes* species mosquito, although other arthropod vectors for Zika virus are possible. Since it was first isolated from a Rhesus monkey in the Zika forest of Uganda in 1947, there were very few reported incidents of human infection, especially outside of the endemic regions of Africa and Asia until a large outbreak in French Polynesia in 2007 (Haddow *et al. PLoS Neglected Tropical Diseases* (2012) 6(2), Malone *et al. PLoS Neglected Tropical Diseases* (2016) 10(3),). The virus has since spread through islands of the Pacific, including Oceania, and into South and Central America (WHO "Zika Situation Report" February 5, 2016).

In addition to being spread by the bite of an infected mosquito, evidence also suggests transmission may occur between individuals, such as from the blood of an infected individual, *in utero*/transplacental transmission from an infected mother to the fetus, sexual transmission between

sexual partners, and possibly by other local transmission routes. There is a possible association between Zika virus infection during pregnancy and microcephaly in the fetus/neonate. Microcephaly is a rare condition in which a baby's head circumference is significantly less than expected based on the average for their age, sex, and ethnicity. This is a result of the brain failing to undergo proper embryonic development, and in 90% of cases is associated with mental retardation (Rocha *et al.* (2016) *Bull World Health Organ* 8 Feb. 2016).

There is a probable association between individuals having had a prior Zika virus infection and the incidence of Guillain-Barré syndrome, a neurological disorder in which the individual's immune system destroys the myelin sheath surrounding axons of the peripheral nervous system (WHO "Zika Situation Report" February 5, 2016).

No specific treatments or vaccines for Zika virus currently exist, and the only measures at this time to prevent infection are through vector control and avoiding travel to regions experiencing outbreaks.

Described herein are Zika virus vaccines and compositions comprising inactivated Zika virus that provide a safe method for generating an immune response to Zika virus, including virus-neutralizing antibodies, that may help prevent against Zika virus infection.

Any strain of Zika virus may be used in the methods and compositions described herein. In some embodiments, the Zika virus is an isolate from an infected subject during a Zika virus outbreak. In some embodiments, the Zika virus is a strain isolated from Africa or from the African virus lineage. In some embodiments, the Zika virus is a strain isolated from Asia or from the Asian lineage (includes also strains from French Polynesia). In some embodiments, the Zika virus is a strain isolated from the Americas (South America, Central America, or North America), such as a Suriname Zika virus strain. In some embodiments, the Zika virus has an RNA genome corresponding (but not limited) to the DNA sequence provided by GenBank Accession No. AY632535.2, KU321639.1, KU497555.1, KU501215.1, KU509998.1, KU527068.1, KU681081.3, KU681082.3, KU707826.1, KU744693.1, or LC002520.1 or RNA genome disclosed partially or fully herein (SEQ ID NO: 2 to 69).

In some embodiments, the process of the invention results in an enrichment of infectious Zika virus particles from the crude harvest comprising infectious Zika virus particles and non-infectious virus particles and other virus products such that the enrichment of the infectious virus particles is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, preferably at least 80%,

especially at least 85% relative to the total virus particle content of the crude harvest (a) comprising the virus particles and impurities.

In some embodiments, the residual impurity of the final virus preparation with respect to all impurities in the crude harvest is less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, preferably less than 5% as determined by SEC-HPLC (Size Exclusion Chromatography – HPLC).

A unique aspect of the current invention is the realization that know-how related to the vaccine design and purification approach used for the Japanese Encephalitis Vaccine (JEV) IXIARO® (see Srivastava A.K. et al., 2001, Vaccine 19, 4557-4565, WO99/11762) may be employed and improved upon in order to expedite the development of a Zika virus vaccine and provide it to the subjects in need as soon as possible. The industrial process as disclosed for IXIARO®, providing a very effective vaccine against JEV, was complemented by further significant improvements disclosed herein in order to provide a more efficient (higher yield) and safer (less or no protamine sulphate with its allergic potential) Zika vaccine compared to the available JEV vaccine. A particular innovation of the herein disclosed vaccines is their greatly reduced protamine salt (SEQ ID NO: 1) content in the final drug substance facilitated by the development of an improved sucrose gradient. Said sucrose gradient not only allowed the separation of protamine sulphate but also allowed for a very effective inactivation by formaldehyde and resulted in the case of Zika with over 90% yield with the improved process disclosed herein vs about 35% yield with the published JEV process, see experimental part for comparison).

Aspects of the disclosure relate to methods of producing a virus in Vero tissue culture cells. Vero cells are a commonly used tissue culture cell line derived from the kidney of an African green monkey. The Vero cells used in the methods described herein are the Vero (WHO) cell line, obtained from the Health Protection Agency general cell collection under catalogue number 88020401.

Vero cells can be grown to confluent monolayers, for example in tissue culture flasks; in suspension (on microcarriers), for example in roller bottles; or in any other cell culture system for viral production. In some embodiments, the Vero cells are grown in a bioreactor for viral production. For plaque assays or the plaque reduction neutralization test (PRNT), Vero cells are grown in monolayers in tissue culture flasks, dishes, or wells of a plate. To infect the Vero cells with the virus, the culture medium is inoculated with virus and the cells are incubated with the virus for a period of time. The cells may be washed after inoculation to remove any virus that did not adsorb to the cells in a given amount of time.

The methods provided herein involve passaging the virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with virus and subsequently inoculating a second population of Vero cells with virus produced by infection of the first Vero cell population. In some embodiments, a portion of the culture medium from the infected Vero cells (containing virus that was released from the infected cells) is used to inoculate a second population of Vero cells. This is referred to as one passage or one round of passaging. The passaging may be performed serially, for example, a portion of the culture medium from the infected second population of Vero cells is used to inoculate a third population of Vero cells, and so on. In some embodiments, virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the virus is passaged in Vero cells several times, such as at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 times. In some embodiments, the virus is passaged in Vero cells at least 4 times or 5 times. In some embodiments, the virus is passaged in Vero cells at least 30 times. It is important that the virus population, i.e. the virus sequences, stays as much as possible constant over said passaging. If adaption of the virus occurs (i.e. appearance of mutated viruses in the original virus population), it is preferred that said passages are not used in the context of manufacturing of said virus, e.g. for Zika it was found that up to passage 3 and culturing to day 7 can be used without major shifts in virus population, i.e. introduction of virus population with mutations. However this observation needs to be done for each virus strain and may be different.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the virus at e.g. a typical 0.01 MOI (multiplicity of infection) to allow for viral production prior to passaging. In some embodiments, the Vero cells are incubated for at least 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 days e.g. at least 7 days after inoculation with the virus prior to passaging. The number of days the Vero cells are incubated after viral inoculation may depend on factors such as the multiplicity of infection used to inoculate the cells and the viral titer desired in the culture medium. Serial passaging of the virus in Vero cells may result in generation of a Vero cell adapted virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the virus. In some embodiments, the culture medium is harvested from infected Vero cells and is replaced with fresh culture medium, which is then harvested after another period of time. In some embodiments, the culture medium harvested from infected Vero cells is pooled from independent Vero cell cultures and/or from independent days. Harvesting can be repeated up to 4 times by 7 or 9 days post infection,

for example, and result in a high yield of virus per unit cell culture. In order to minimize the adaption of Zika virus strain to Vero cells, it was found that Vero cells could be incubated for at least 7 days, more preferably 5 days, prior to passaging and subsequently supernatants could be harvested at days 2, 3, 5 and 7 or 2, 3, and 5 (see also experimental part). The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium up to 2 weeks.

In some embodiments, debris from infected and lysed Vero cells may be removed from the harvested culture medium, referred to as a "clarification" of the culture medium. The harvested culture medium may be clarified by common methods known in the art, such as low-speed centrifugation, for example, at 1500 *g* for 10 min, and/or by filtration through a filter of pore size of 0.45 µm. The harvested culture medium can be stored at +4°C prior to concentration.

To concentrate the titer of the Zika virus in the harvested culture medium, it may be subjected to concentration by any method known in the art. For example, the harvested culture medium may be concentrated by methods including, without limitation, ultrafiltration, ultracentrifugation, centrifugal concentrator, vacuum centrifugation, and lyophilization. In some embodiments, the harvested culture medium is concentrated by ultrafiltration and the retentate containing the Zika virus is collected. In some embodiments, the harvested culture medium is concentrated by precipitation in which polyethylene glycol (PEG) 8000 is dissolved in the culture medium (up to 10%) and the precipitate is dissolved in a buffer, for example phosphate-buffered saline (PBS, pH 7.0).

The harvested culture medium may be precipitated to produce a virus supernatant. In some embodiments, the harvested culture medium is precipitated to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. In some embodiments, the harvested culture medium is concentrated prior to precipitation. In some embodiments, the harvested culture medium is precipitated by adding protamine sulfate (e.g. SEQ ID NO: 1) to the harvested culture medium and incubating the mixture, for example at +4°C or on ice. In some embodiments, the harvested culture medium is treated with benzonase to remove Vero cell DNA and other undesired material, such as Vero cell debris, from the harvested culture medium. However, it was found that the treatment with protamine sulfate is preferred (see experimental part). In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the virus, referred to as a "virus supernatant", is collected.

The virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the virus supernatant is further purified by sucrose

gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the virus. Methods for assaying for virus positive fractions include plaque assay, hemagglutination assay, polyacrylamide gel electrophoresis, and antigen assays such as Western blotting and ELISA. The fractions containing virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the virus supernatant can be estimated by testing for Vero cell DNA, virus aggregates and/or Vero cell protein (see experimental part). A particular embodiment of the invention is the improved sucrose gradient that allows for an efficient protamine separation as shown in the experimental part. It was surprisingly found that the addition of a virus-containing fraction with 10% (w/w) sucrose to a simple three layer sucrose density gradient (e.g. a gradient comprising a 15% (w/w) sucrose solution, a 35% (w/w) sucrose solution, and a 50% (w/w) sucrose solution) resulted in efficient separation of protamine sulphate without much loss of virus. Thus a particularly preferred embodiment of the invention is the use of a sucrose density gradient that is able to efficiently separate protamine sulphate, wherein said sucrose density gradient is used in the purification of virus such as the viruses described herein, i.e. a Zika virus.

To achieve a safe vaccine or composition for the administration to subjects, the virus supernatant may be inactivated (see experimental part for Zika virus). As used herein, the terms "inactivated" and "optimally inactivated" may be used interchangeably and refer to a process (or its result) by which the virus is rendered unable to infect a host cell (non-infectious), but that does not affect or substantially affect the antigenicity of the virus, for example, the immunogenic antigens exposed on the surface of the virus are able to stimulate an immune response in a subject (*e.g.*, antigen-specific antibodies). By "does not affect or substantially affect the antigenicity of the virus" is meant that the inactivated virus retains at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even essentially 100% of the antigenicity of a virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

In some embodiments, the inactivating is by chemical inactivation and involves contacting the virus with one or more chemical inactivation agents for a period of time under conditions such that the virus is inactivated but the antigenic epitopes are substantially intact. In some embodiments, the virus is inactivated for a period of time that is longer than is required to completely inactivate the virus. In some embodiments, the virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the virus supernatant may be taken at one or

more times throughout the inactivation process and assessed for viral viability (infectivity) by any method known in the art, such as by infecting a monolayer of host cells (*i.e.*, plaque assay). Using such a procedure, the period of time that is required to completely inactivate the virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the virus is contacted with a chemical inactivation agent for between 1 day and 50 days, between 2 days and 40 days, between 2 days and 30 days, between 2 days and 20 days, between 2 days and 10 days, between 3 days and 9 days, between 4 days and 8 days, between 5 days and 7 days, between 2 days and 5 days, or between 5 and 10 days. In some embodiments, the virus is contacted with one or more chemical inactivation agents for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, or at least 50 days.

In some embodiments, the chemical inactivation is performed at about +5°C, +10°C, +15°C, +20°C, +25°C, +30°C, +35°C, +40°C, or about +45°C. In some embodiments, the chemical inactivation is performed at about +4°C. In some embodiments, the chemical inactivation is performed at about +22°C.

Any chemical inactivation agent known in the art may be suitable for inactivating the virus in the methods described herein. It will be appreciated by one of skill in the art that factors such as the chemical inactivation agent and the temperature at which inactivation is performed may affect the length of time (number of days) required to completely inactivate the virus. Examples of chemical inactivation agents include, without limitation, formaldehyde, enzymes, β-propiolactone, ethanol, trifluroacetic acid, acetonitrile, bleach, urea, guanidine hydrochloride, tri-n-butyl phosphate, ethylene-imine or a derivatives thereof, and organic solvents such as Tween, Triton, sodium deoxycholate, and sulphobetaine. A preferred inactivation is inactivation with formaldehyde at 22°C +/-2°C for about 10 days.

In some embodiments, the inactivating agent is neutralized after chemical inactivation of the virus. In some embodiments, the inactivating agent is formaldehyde and is neutralized after chemical inactivation using sodium thiosulphate or sodium metabisulfite.

In some embodiments, the virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the virus to heat, such as dry heat or vapor heat, for a period of time. In some embodiments, the thermal inactivation involves exposing the virus to temperatures of about +40°C, +45°C, +50°C, +55°C, +60°C, +65°C, +70°C, +75°C, +80°C, +85°C, +90°C,+ 95°C, or about +100°C. In some embodiments, the virus is exposed to heat for at least 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, about 96 hours, or longer. A preferred thermal inactivation involves exposing the virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the virus is inactivated by exposing the virus to acidic or alkaline conditions for a period of time such that the virus is completely inactivated. The pH of a virus preparation may be adjusted to a desired pH, for example by the addition of an acid, a base, or a buffer with a particular pH to the virus preparation. In some embodiments, the virus is inactivated at an acidic pH of about 2, 2.5, 3, 3.5, 4, 4.5, 5 or about 5.5. In other embodiments, the virus is inactivated at an alkaline pH of about 8, 8.5, 9, 9.5, 10, or about 10.5.

In some embodiments, the virus is inactivated using UV inactivation. UV inactivation involves exposing the virus to energy-rich radiation, such as UV-A, UV-B, or UV-C light for a period of time. It will be appreciated that any two or more methods of inactivation may be combined and performed concurrently or serially.

The inactivated virus may be subsequently dialyzed to remove any undesired material, including the inactivating agent and any neutralizing agent, and/or to replace the buffer with a buffer that is pharmaceutically acceptable for administration to subjects. In some embodiments, the inactivated virus is dialyzed with PBS. In addition or alternatively, the inactivated virus may be filtered, such as sterile filtered, through a 0.22 μm filter.

Any of the methods or uses described herein may be for the prevention of a Zika virus infection in a subject. As used herein, the terms "prevent," "preventing" and "protection from" include the administration of a virus vaccine or composition to a subject to reduce, or delay the onset of the manifestation of clinical or subclinical symptoms, complications, pathologies or biochemical indicia of a disease or infection, or to reduce or inhibit the spread/transmission of the Zika virus. As used herein, antigen(s), such as an inactivated Zika virus, that is administered to a subject prophylactically (e.g., prior to infection) may be referred to as a vaccine.

Zika Vaccine

As described herein Zika virus may cause any of a variety of symptoms upon infection of a subject, and is generally characterized by mild fever; rash (exanthema) on face, neck, trunk, upper arms; headache; sensitivity to light; non-inflammatory joint pain; conjunctivitis; lack of appetite; diarrhea; abdominal pain; and/or dizziness. Zika virus infection during pregnancy is associated with microcephaly in the fetus/neonate. There is also a probable association between the onset of Guillain-Barré syndrome or symptoms thereof. Diagnosis of Zika virus infection in subjects exposed to Zika virus or suspected of being exposed to Zika virus involves detecting the presence of virus-specific antibodies and/or molecular testing, such as PCR or real-time PCR detection of Zika virus.

Provided herein are methods for administering a dose of a therapeutically effective amount of a Zika virus vaccine to a subject in need thereof. In some embodiments, the subject is a mammalian subject, such as a human, non-human primate, rodent, rabbit, sheep, dog, cat, horse, or cow. In some embodiments, the subject is a mouse. In some embodiments, the subject is a human subject, such as a child, an adult, or an elderly adult. In some embodiments, the subject is a female subject. In some embodiments, the subject is pregnant or planning on becoming pregnant. In some embodiments, the subject is at risk of being exposed to Zika virus. In some embodiments, the subject is living in or traveling to an area where Zika virus is present or is thought to be present. In some embodiments, the subject has been previously infected with or vaccinated against Dengue virus; i.e., at risk for antibody-dependent enhancement of disease. In some embodiments, the subject is living in or traveling to an area that is experiencing a Zika virus infection outbreak. In some embodiments, the subject is living in or traveling to an area where an arthropod vector capable of transmitting the Zika virus vector is present or is thought to be present.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject in a therapeutically effective amount or a dose of a therapeutically effective amount. As used herein, a "therapeutically effective amount" of vaccine is any amount that results in a desired response or outcome in a subject, such as those described herein, including but not limited to prevention of infection, an immune response or an enhanced immune response to Zika virus, or prevention or reduction of symptoms associated with Zika disease.

In some embodiments, the therapeutically effective amount of a Zika virus vaccine or composition described herein is an amount sufficient to generate antigen-specific antibodies (*e.g.*, anti-Zika virus

antibodies). In some embodiments, the therapeutically effective amount is sufficient to provide seroprotection in a subject; i.e., to generate sufficient antigen-specific antibodies to prevent/protect from infection. In some embodiments, seroprotection is conferred on at least 75%, 80%, 90%, 95%, 96%, 97%, 98%, or at least 99% of vaccinated subjects. In some embodiments, seroprotection is defined by a reduction in the number of Zika virus plaques by 50% or more in a plaque reduction neutralization test (PRNT) by a 1:10 or higher dilution of sera from a vaccinated subject. In some embodiments, an effective amount of the Zika vaccine is sufficient to seroconvert a subject with at least 70% probability. In some embodiments, the therapeutically effective amount is sufficient to seroconvert a subject with at least 75%, 80%, 85% 90%, 95%, 96%, 97%, 98%, or at least 99% probability. Whether a subject has been seroconverted can be assessed by any method known in the art, such as obtaining a serum sample from the subject and performing an assay to detect anti-Zika virus antibodies. In some embodiments, a subject is seroconverted if a serum sample from the subject contains an amount of anti-Zika virus antibodies that surpasses a threshold or predetermined baseline. A subject is generally considered seroconverted if there is at least a 4-fold increase in anti-Zika virus antibodies (*i.e.,* anti-Zika E protein IgG antibodies) in a serum sample from the subject as compared to a serum sample previously taken from the same subject.

In some embodiments, seroconversion of a subject is assessed by performing a plaque reduction neutralization test (PRNT). Briefly, PRNT is used to determine the serum titer required to reduce the number of Zika virus plaques by 50% (PRNT50) as compared to a control serum/antibody. The PRNT50 may be carried out using monolayers of Vero cells or any other cell type/line that can be infected with Zika virus. Sera from subjects are diluted and incubated with live, non-inactivated Zika virus. The serum/virus mixture may be applied to the Vero cells and incubated for a period of time. Plaques formed on the Vero cell monolayers are counted and compared to the number of plaques formed by the Zika virus in the absence of serum or a control antibody. A threshold of neutralizing antibodies of 1:10 dilution of serum in a PRNT50 is generally accepted as evidence of protection (Hombach *et. al.* Vaccine (2005) 23:5205-5211).

In some embodiments, the Zika virus may be formulated for administration in a composition, such as a pharmaceutical composition. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of at least one active ingredient, such as an inactivated Zika virus, and one or more inactive ingredients, which may include one or more pharmaceutically acceptable excipient.

Pharmaceutical compositions of the invention, including vaccines, can be prepared in accordance with methods well known and routinely practiced in the art (see *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co. 20th ed. 2000; and Ingredients of Vaccines – Fact Sheet from the Centers for Disease Control and Prevention, e.g., adjuvants and enhancers such as alum to help the vaccine improve its work, preservatives and stabilizers to help the vaccine remain unchanged (e.g., albumin, phenols, glycine)). Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically a therapeutically effective dose of the inactivated Zika virus preparation is employed in the pharmaceutical composition of the invention. The inactivated Zika virus is formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, the prophylactic response).

Dosages of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired pharmaceutical response for a particular subject, composition, and mode of administration, without being toxic to the subject. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors.

A physician, veterinarian or other trained practitioner, can start doses of the inactivated Zika virus vaccine employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect (*e.g.*, production of anti-Zika virus antibodies) is achieved. In general, effective doses of the compositions of the present invention, for the prophylactic treatment of groups of people as described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and the titer of anti-Zika virus antibodies desired. Dosages need to be titrated to optimize safety and efficacy. In some embodiments, the dosing regimen entails subcutaneous or intramuscular administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 7. In some embodiments, the dosing regimen entails subcutaneous administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 14. In some embodiments, the dosing regimen entails subcutaneous

administration of a dose of inactivated Zika virus twice, once at day 0 and once at about day 28. In some embodiments, the inactivated Zika virus is administered to the subject once.

Any of the Zika virus vaccines or compositions described herein may be administered to a subject with, prior to, or after administration of one or more adjuvants. An adjuvant is a molecule that enhances a response in a subject, such as an immune response, to an antigen or other molecule. In some embodiments, an adjuvant may stabilize an antigen or other molecule. Determining whether a Zika virus vaccine or compositions thereof are administered with an adjuvant depends on various factors (e.g., type and extent of response desired) and will be evident to one of skill in the art. In some embodiments, administering any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may enhance the production of virus neutralizing (anti-Zika virus) antibodies. In some embodiments, a subject that is administered any of the Zika virus vaccines or compositions described herein with, prior to, or after administration of an adjuvant may only require a single administration of the Zika virus vaccine or composition to be seroconverted (produce a level of anti-Zika virus antibodies). Examples of adjuvants may include, without limitation, aluminium salt (aluminium hydroxide or aluminium phosphate), calcium phosphate hydroxide, paraffin oil, killed bacteria, bacterial toxins, toxoids, subunits of bacteria, squalene, thimerosal, detergents, IL-1, IL-2, IL-12, 2-component adjuvants, such as 2-component adjuvants containing an antibacterial peptide and a TLR9 agonist (*e.g.*, IC31®), and combinations such as Freund's complete adjuvant and Freund's incomplete adjuvant. In some embodiments, the Zika virus vaccines or compositions is administered with aluminium hydroxide. In some embodiments, the inactivated Zika virus vaccine or composition is administered with aluminium phosphate salt. A preferred aluminium salt is the aluminium hydroxide with reduced Cu content, e.g. lower than 1,25ppb based on the weight of the Zika composition, an adjuvant described in detail in WO 2013/083726 or Schlegl et al., Vaccine 33 (2015) 5989-5996.

In some embodiments, the adjuvant is comprised of two components. In some embodiments, the 2-component adjuvant comprises an antibacterial peptide and a TLR9 agonist. In some embodiments, the antibacterial peptide is provided by the amino acid sequence $KLKL_5KLK$ (SEQ ID NO: 71). In some embodiments, the TLR9 agonist is a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN). In some embodiments, the I-ODN comprises the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70). In some embodiments, the adjuvant is IC31®. In some embodiments, the adjuvant is in nanoparticle form (See, *e.g.*, US Patent No. 8,765,148 B2, incorporated by reference in its entirety). In some embodiments, the adjuvant is IC31®, i.e.

$KLKL_5KLK$ (SEQ ID NO: 71) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 70), in combination with an aluminium salt such as aluminium hydroxide.

The Zika virus vaccines or compositions described herein may be administered to a subject concomitantly with one or more vaccines to another infectious agent, such as another infectious agent is that present or thought to be present in the same geographic area as Zika virus. In some embodiments, the other infectious agent is one that the subject is also at risk of being in contact with. In some embodiments, the other infectious agent is transmitted by the same arthropod vector as Zika virus. In some embodiments, the other infectious agent is Japanese Encephalitis virus, Yellow Fever virus, Dengue virus and/or Chikungunya virus.

Also within the scope of the present disclosure are kits for use in prophylactically administering to a subject, for example to prevent or reduce the severity of Zika virus infection. Such kits can include one or more containers comprising a composition containing inactivated Zika virus, such as an inactivated Zika virus vaccine. In some embodiments, the kit may further include one or more additional containing comprising a second composition, such as a second vaccine. In some embodiments, the second vaccine is a vaccine for another arbovirus. In some embodiments, the second vaccine is a Dengue virus vaccine and/or a Chikungunya virus vaccine.

In some embodiments, the kit can comprise instructions for use in accordance with any of the methods described herein. The included instructions can comprise a description of administration of the composition containing inactivated Zika virus to prevent, delay the onset, or reduce the severity of Zika virus infection. The kit may further comprise a description of selecting a subject suitable for administration based on identifying whether that subject is at risk for exposure to Zika virus or contracting a Zika virus infection. In still other embodiments, the instructions comprise a description of administering a composition containing inactivated Zika virus to a subject at risk of exposure to Zika virus or contracting Zika virus infection.

The instructions relating to the use of the composition containing inactivated Zika virus generally include information as to the dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine readable instructions are also acceptable.

The kits of the present disclosure are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as a syringe or an infusion device. The container may have a sterile access port, for example the container may be a vial having a stopper pierceable by a hypodermic injection needle. At least one active agent in the composition is an inactivated Zika virus, as described herein.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having,", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Table 1. Overview of process buffers and stock solutions.

Buffer	Composition	Final pH	Final conductivity [mS/cm]
A	0.5 M NaOH		n.a.
B	0.1 M NaOH		n.a.
C	25 mM Tris, 150 mM NaCl	7.4±0.2	16.5
D	1 M Tris	7.4±0.2	n.a.
E	4.5 M NaCl	n.a.	n.a.
F	1 M NaCl	n.a.	n.a.
G	1 % SDS	n.a.	n.a.
H	50 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
I	35 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
J	15 % (w/w) Sucrose in 25 mM Tris, 150 mM NaCl	7.4±0.2	n.a.
K	10 x PBS	7.4±0.2	n.a.
L	50 mg/mL Protamine sulphate	7.4±0.2	n.a.
M	Drug substance formulation buffer (10mM Tris(hydroxymethyl)-aminomethan, 5 % Sucrose, 1% (10 mg/mL) rHSA)	7.4±0.2	1.3

Table 2. Abbreviations.

°Bx	Degrees Brix = sugar content (w/w) of an aqueous solution*
BSA	Bovine serum albumin
CC700	Capto™ Core 700
CPE	Cytopathic effect
EtOH	Ethanol
EU	Endotoxin units
DS	Drug Substance
DP	Drug Product
DSP	Downstream Process
HCP	Host cell protein
hcDNA	Host cell DNA
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
ID	Inner diameter
JEV	Japanese Encephalitis virus
LAL	Limulus amebocyte lysate
LDS buffer	Lithium dodecyl sulfate sample loading buffer
LOD	Limit of detection
LOQ	Limit of quantitation
MALLS	Multiangle light scattering
mAU	Milli absorbance units
MS	Mass spectroscopy
NIV	Neutralized inactivated virus
PBS	Phosphate buffered saline
PD	Process development
PFU	Plaque forming units
p.i.	Post-infection
PS	Protamine sulphate or protamine sulfate
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin
Rms radius	Root mean square radius
rMSB	Research master seed bank
RSD	Relative standard deviation
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation

SGP	Sucrose gradient purified
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
WFI	Water for injection
ZikaV	Zika virus

*Degrees Brix (°Bx) is the sugar content of an aqueous solution. One degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. °Bx corresponds to the sucrose content in percent (w/w), e.g., 45 °Bx equals 45 % (w/w) sucrose.

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Table A. Primers for Zika virus sequencing: lower case letters indicate bases not included in ZIKA but containing restriction sites for later cloning when needed (therefore, two Tms provided).

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
1	9320_Zika_PF_1F	SEQ ID NO: 74 ttaggatccGTTGTTGATCTGTGTGAAT	69.9	74.6	707
	9321_Zika_PF_1R	SEQ ID NO: 75 taactcgagCGTACACAACCCAAGTT	69.3	75.6	
2	9322_Zika_PF_2F	SEQ ID NO: 76 ttaggatccTCACTAGACGTGGGAGTG	70	73.9	704
	9323_Zika_PF_2R	SEQ ID NO: 77 taactcgagAAGCCATGTCYGATATTGAT	69.8	73.7	
3	9324_Zika_PF_3F	SEQ ID NO: 78 ttaggatccGCATACAGCATCAGGTG	72.3	74.5	712
	9325_Zika_PF_3R	SEQ ID NO: 79 taactcgagTGTGGAGTTCCGGTGTCT	72	76.4	
4	9326_Zika_PF_4F	SEQ ID NO: 80 ttaggatccGAATAGAGCGAARGTTGAGATA	70.9	74	712
	9327_Zika_PF_4R	SEQ ID NO: 81 taactcgAGTGGTGGGTGATCTTCTTCT	70.5	73.7	
5	9328_Zika_PF_5F	SEQ ID NO: 82 ttaggatcCAGTCACAGTGGAGGTACAGTAC	70.3	75	704
	9329_Zika_PF_5R	SEQ ID NO: 83 taactcgagCRCAGATACCATCTTCCC	71.5	77.3	
6	9330_Zika_PF_6F	SEQ ID NO: 84 ttaggatCCCTTATGTGCTTGGCCTTAG	70.7	72.7	698
	9331_Zika_PF_6R	SEQ ID NO: 85 taactcgagTCTTCAGCCTCCATGTG	70.4	76.9	
7	9332_Zika_PF_7F	SEQ ID NO: 86 ttaggatccAATGCCCACTCAAACATAGA	71.9	75	716
	9333_Zika_PF_7R	SEQ ID NO: 87 taactcgagTCATTCTCTTCTTCAGCCCTT	71	74	
8	9334_Zika_PF_8F	SEQ ID NO: 88 ttaggatccAAGGGTGATCGAGGAAT	70.9	75.2	703
	9335_Zika_PF_8R	SEQ ID NO: 89 taactcgagTTCCCTTCAGAGAGAGGAGC	71.9	73.4	
9	9336_Zika_PF_9F	SEQ ID NO: 90 ttaggatccTCTTTTGCAAACTGCGATC	71.9	75	699
	9337_Zika_PF_9R	SEQ ID NO: 91 taactcgagTCCAGCTGCAAAGGGTAT	71	74.9	
10	9338_Zika_PF_10F	SEQ ID NO: 92 ttaggatccGTGTGGACATGTACATTGA	71.4	75.8	706
	9339_Zika_PF_10R	SEQ ID NO: 93 taactcgagCCCATTGCCATAAAGTC	70.4	75.8	

Primer Pair	Oligoname	Primer sequence (5'-3') restriction sites (lower case)	Tm (gene-specific)	Tm (entire primer)	Amplicon size [bp]
11	9340_Zika_PF_11F	SEQ ID NO: 94 ttaggatccTCATACTGTGGTCCATGGA	71.6	78.1	692
	9341_Zika_PF_11R	SEQ ID NO: 95 taactcgagGCCCATCTCAACCCTTG	74	78	
12	9342_Zika_PF_12F	SEQ ID NO: 96 ttaggatccTAGAGGGCTTCCAGTGC	70.9	74	707
	9343_Zika_PF_12R	SEQ ID NO: 97 taactcgAGATACTCATCTCCAGGTTTGTTG	70.2	72.2	
13	9344_Zika_PF_13F	SEQ ID NO: 98 ttaggatccGAAAACAAAACATCAAGAGTG	70.6	75.4	726
	9345_Zika_PF_13R	SEQ ID NO: 99 taactcgagGAATCTCTCTGTCATGTGTCCT	71.9	75.6	
14	9346_Zika_PF_14F	SEQ ID NO: 100 ttaggatccTTGATGGCACGACCAAC	73.1	75.6	715
	9347_Zika_PF_14R	SEQ ID NO: 101 ttaggatccGTTGTTGATCTGTGTGAAT	70.8	77.9	
15	9348_Zika_PF_15F	SEQ ID NO: 102 taactcgagCAGGTCAATGTCCATTG	71.9	75.4	719
	9349_Zika_PF_15R	SEQ ID NO: 103 ttaggatccTGTTGTGTTCCTATTGCTGGT	73.9	77.2	
16	9350_Zika_PF_16F	SEQ ID NO: 104 taactcgaGTGATCAGRGCCCCAGC	72.3	75.4	703
	9351_Zika_PF_16R	SEQ ID NO: 105 ttaggatccTGCTGCCCAGAAGAGAA	72	76.3	
17	9352_Zika_PF_17F	SEQ ID NO: 106 taactcgaGCACCAACAYGGGTTCTT	73.6	76	705
	9353_Zika_PF_17R	SEQ ID NO: 107 ttaggatcCTCAAGGACGGTGTGGC	72	75.5	
18	9354_Zika_PF_18F	SEQ ID NO: 108 taactcgagCAATGATCTTCATGTTGGG	71.7	75.8	699
	9355_Zika_PF_18R	SEQ ID NO: 109 ttaggatccTATGGGGGAGGACTGGT	71	74.1	
19	9356_Zika_PF_19F	SEQ ID NO: 110 taactcGAGCCCAGAACCTTGGATC	73.3	75.5	711
	9357_Zika_PF_19R	SEQ ID NO: 111 ttaggatcCAGACCCCCAAGAAGGC	71.3	76.9	
20	9358_Zika_PF_20F	SEQ ID NO: 112 taactcgagCCCCTTTGGTCTTGTCT	71.7	75	706
	9359_Zika_PF_20R	SEQ ID NO: 113 ttaggatccAGGAAGGATGTATGCAGATG	71.9	73.9	
21	9360_Zika_PF_21F	SEQ ID NO: 114 taactcgagACATTTGCGCATATGATTTTG	70.4	75.7	709
	9361_Zika_PF_21R	SEQ ID NO: 115 ttaggatccAGGAAGGACACACAAGAGT	71.8	75	
22	9362_Zika_PF_22F	SEQ ID NO: 116 taactcgagACAGGCTGCACAGCTTT	70	79.1	581
	9363_Zika_PF_22R	SEQ ID NO: 117 ttaggatccTCTCTCATAGGGCACAGAC	74.8	81.1	

SEQUENCES

SEQ ID NO: 1
A typical form of protamine
PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR

Provided below are examples of nucleic acid sequences of the genomes of Zika viruses that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 2

KU321639.1 Zika virus strain ZikaSPH2015, Brazil, complete genome

GTTGTTACTGTTGCTGACTCAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTGG
ATTTGGAAACGAGAGTTTCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACG
CGGAGTAGCCCGTGTGAGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGG
ATGGTCTTGGCAATTCTAGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTG
GGGAAAAAAGAGGCTATGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGA
AGGAGAAGAAGAGACGGGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGG
TCACTAGACGTGGGAGTGCATACTATATGTACTTGGACAGAAACGATGCTGGGGAGGCCATATCTTTTCCAACCACATTG
GGGATGAATAAGTGTTATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCT
GGATGAGGGGGTGGAACCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCAT
CACAAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGT
CGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTC
GCGTTAGCAGCAGCTGCCATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCT
GATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGG
GTTGATATTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTAC
AACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCAGACATGGCTTCGGACAGCCGCT
GCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGC
TGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGTCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCG
GGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGT
TAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACC
CTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATG
AATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAAC
TCCACACTGGAACAACAAAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGG
AGTCAAGAAGGAGCAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCT
CTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCA
CATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTG
CAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCA
CTGAAAGCACTGAGAACTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGG
GAGAAGAAGATCACCCACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTGAAGCCACTGTGAGAGGTGCCA
AGAGAATGGCAGTCTTGGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCTCAACTCATTGGGCAAGGGCAT
CCATCAAATTTTTGGAGCAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACAAATTCTCATTGGAACGTTGCTG
ATGTGGTTGGGTCTGAACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGGGGGAGTGTTGATCTTCTTATCC
ACAGCCGTCTCTGCTGATGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGATGCGGTACAGGGGTGTTCGTCT
ATAACGACGTTGAAGCCTGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGATTGGCAGCAGCAGTCAAGCA
AGCCTGGGAAGATGGTATCTGCGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTGGAGATCAGTAGAAGGGGAG
CTCAACGCAATCCTGGAAGAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTC
CACAGAGATTGCCCGTGCCTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGCACTTCGTCAGAGCAGC
AAAGACAAATAACAGCTTTGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTC
TTGTGGAGGATCATGGGTTCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGAT
CCAGCCGTTATTGGAACAGCTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGA
ATGACACATGGAGGCTGAAGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGAC
AGATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCT
ACAGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCA
CGTGGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGATCGAGGAATGGTG
CTGCAGGGAGTGCACAATGCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGAATGGAGATAAGGCCCAGG
AAAGAACCAGAAAGCAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGT
GCTTGTGATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCAATGGCA
GTGCTGGTAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTTGATGGGTGCCACCTTCGC
GGAAATGAACACTGGAGGAGATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGACCAGCGTTGCTGGTATCTT

TCATCTTCAGAGCTAATTGGACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTTTGCAAACTGCGATCTCCG
CCTTGGAAGGCGACCTGATGGTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCA
CTGATAACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGGAGAGCAGGC
CTTGCTACTTGCGGGGGGTTTATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAACTTACCATTTGTCATGGC
CCTGGGACTAACCGCTGTGAGGCTGGTCGACCCCATCAACGTGGTGGGGCTGCTGTTGCTCACAAGGAGTGGGAAGCGG
AGCTGGCCCCCTAGCGAAGTACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTCGCCAAGGCAGATATAGA
GATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGAGTGTGGACATGTACATTG
AAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCGATGTGGCGCTAGATG
AGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAGGTGGTCCTGATGACCATC
TGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACTGGAAAAAGGAGTGGTG
CTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAG
ACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACAAAAGGA
TCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGTCATACTGTGGTC
CATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGA
ACATCCAGACTCTGCCCGGAATATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAAC
TTCAGGATCTCCAATCCTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGT
TATGTTAGTGCCATCACCCAAGGGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGCCTTCGATGCTGAAGAAGA
AGCAGCTAACTGTCTTAGACTTGCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAAATAGTCCGTGAAGCCATA
AAAACAAGACTCCGTACTGTGATCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTTCC
AGTGCGTTATATGACAACAGCAGTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAATGTGCCATGCCACCTTCAC
TTCACGTCTACTACAGCCAATCAGAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCACTTCACAGATCCCTCAAGT
ATAGCAGCAAGAGGATACATTTCAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCATGACCGCCACGCCACCAG
GAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCA
GGCTTTGATTGGGTGACGGATTATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAACGGCAATGAGATCGCAGC
TTGTCTGACAAAGGCTGGAAAACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGTTCCAGAAAACAAAACATC
AAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGACCGTGTCATAGATTCCAGG
AGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGTCACACATGCCAGCGCTGC
CCAGAGGAGGGGGCGCATAGGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGAC
TGACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGATGGCCTCATAGCCTCGCT
CTATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACCTTTGTG
GAACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAATAACCTACACAGATAGAAG
ATGGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTGTGGACCAGACACGGAGA
GAAAAGAGTGCTCAAACCGAGGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGAAGTCATTCAAGGAGTTT
GCCGCTGGGAAAAGAGGAGCGGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACACATGACAGAGAGATTC
CAGGAAGCCATTGACAACCTCGCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAAGCCGCGGCGGCCCAAT
TGCCGGAGACCCTAGAGACCATTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTTTTTCGTCTTGATGAGG
AACAAGGGCATAGGGAAGATGGGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATGTGGCTCTCGGAAATTG
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SEQ ID NO: 3
KU497555.1 Zika virus isolate Brazil-ZKV2015, Brazil, complete genome
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SEQ ID NO: 4
KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome
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SEQ ID NO: 6

KU527068.1 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, complete genome

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SEQ ID NO: 7
KU681081.3 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, complete genome
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KU707826.1 Zika virus isolate SSABR1, Brazil, complete genome

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KU744693.1 Zika virus isolate VE_Ganxian, China, complete genome
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SEQ ID NO: 11

LC002520.1 Zika virus genomic RNA, strain: MR766-NIID, Uganda, complete genome

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SEQ ID NO: 12
AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome
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SEQ ID NO: 13
KJ776791.1, Zika virus strain H/PF/2013 polyprotein gene, complete cds
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TGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGTCCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAG
CCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTAC
CGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAAC
CATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCA
GCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGT
ATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGGAGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACC
CCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGC
GTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACAC
AATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGT
CGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTGATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGA
ACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTA
CACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGGGGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGG
CCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCC
CGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCATGCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTG
GAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATCTTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCA
CCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGGAGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTA
TGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTCTTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTG
ACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGCACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTT
GAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCCCATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCA
GCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCG
AAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGCTCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGA
AATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGGCTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCAT
TGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGG
GCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAA
ACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATGACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAG
GAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCACTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGA
AAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAAGAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATT
AGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAGTGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGT
GGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGAGTTGTGTGTACAACATGATGGGAAAAAGAGAAAAGA
AACAAGGGGAATTTGGAAAGGCCAAGGGCAGCCGCGCCATCTGGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGA
AGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGAGAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACA
AAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGAC
ACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCAT
TGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGA
CATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGC
AACTCATTCGGAATATGGAGGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGAC

CAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATT
GATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGGAAAAGTTAGGAAGGACACACAAGAGTGGAAACCCT
CAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCACCACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCC
ATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGCCCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGG
AGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTCCTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCA
ATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGGAGAACTACCTGGTCAATCCATGGAAAGGGAGAATGG
ATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGATTGAGGAGAACGACCACATGGAAGACAAGACCCCA
GTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGACTTGTGGTGTGGATCTCTCATAGGGCACAGACCGC
GCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGA
CTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACACCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTC
AGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGTGACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTA
TAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACC
CCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGAT
CAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAG

In some embodiments, the Zika virus has a RNA genome corresponding to the DNA sequence provided by the nucleic acid sequence of any one of SEQ ID NOs: 2-13 or 72. In some embodiments, the Zika virus has a variant genome that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-13 or 72.

Provided below are amino acid sequences of the E-proteins of Zika strains that may be used in the methods, compositions, and/or vaccines described herein.

SEQ ID NO: 14
isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 15
isol-IbH_30656.AEN75265.1.Nigeria/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADTETPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
RDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 16
ArB1362.AHL43500.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXXXXXXXNR

AEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 17
ArD128000.AHL43502.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMXXXXXGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 18
ArD158095.AHL43505.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 19
ArD158084.AHL43504.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 20
isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 21
isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 22
isol-ArD_41519.AEN75266.1.Senegal/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVE
FKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 23
MR766-NIID.BAP47441.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 24
LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMTVNDIGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 25
isol-MR_766.AEN75263.1.Uganda/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGYETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 26
ArD7117.AHL43501.1.-/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAVCTAAKVPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKA
FEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGG
VMIFLSTAVSA

SEQ ID NO: 27
AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE

VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 28
MR_766.AAV34151.1.Uganda/291-790 Flavivirus envelope glycoprotein E. |Q32ZE1|Q32ZE1_9FL
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 29
MR_766.YP_009227198.1.Uganda/1-500 envelope protein E [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIGYETDEDRAKVE
VTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAH
AKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQY
AGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATV
RGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFL
STAVSA

SEQ ID NO: 30
KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 31
isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHTGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 32
CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 Flavivirus envelope glycoprotein E. (Fragment) OS=Zika virus GN=E PE=4 SV=1
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV

EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 33
Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 34
isol-Si323.AMC37200.1.Colombia/1-504 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 35
KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 36
KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 37
isol-GDZ16001.AML82110.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 38

60

BeH819015.AMA12085.1.Brazil/291-794 Flavivirus envelope glycoprotein E.]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 39
MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 40
KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 41
Haiti%1225%2014.AMB37295.1.Haiti/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 42
KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 43
isol-Z1106027.ALX35662.1.Suriname/5-508 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF

KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 44
isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 45
PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 46
BeH818995.AMA12084.1.Brazil/291-794 Flavivirus envelope glycoprotein E. [Zika virus].
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 47
H/PF/2013.AHZ13508.1.French_Polynesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 48
PRVABC59.AMC13911.1.Puerto_Rico/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 49
KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 50
ZikaSPH2015.ALU33341.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 51
103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 52
isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 53
KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 54
isol-ZJ03.AMM39806.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN

RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 55
isol-FSS13025.AFD30972.1.Cambodia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 56
isol-Z1106032.ALX35660.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 57
isol-Z1106033.ALX35659.1.Suriname/291-794 Flavivirus envelope glycoprotein E. [Zika virus]
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 58
isol-BeH828305.AMK49165.1.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 59
isol-GD01.AMK79468.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNGTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 60
isol-Z1106031.ALX35661.1.Suriname/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSA

SEQ ID NO: 61
ACD75819.1.Micronesia/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 62
KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 63
isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 64
isol-BeH823339.AMK49164.2.Brazil/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCPTQGEAYLD
KQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENR
AKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFK
DAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVE
VQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFE
ATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGV
LIFLSTAVSA

SEQ ID NO: 65
isol-P6-740.AEN75264.1.Malaysia/291-794 Flavivirus envelope glycoprotein E.

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDXGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTV
EVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
LIFLSTAVSA

SEQ ID NO: 66
KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 67
isol-VE_Ganxian.AMK79469.1.China/291-794 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSGMLVNDTGHETDEN
RAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPWHAGAATGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETVDGTVTV
EGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIGAAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGG
VLIFLSTAVSG

SEQ ID NO: 68
ArD157995.AHL43503.1.-/291-794 Flavivirus envelope glycoprotein E.
ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCPTQGEPSL
DKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSGMIVNDIGHETDENR
AKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEF
KDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTV
EVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAF
EATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGV
MIFLSTAVSA

SEQ ID NO: 69
MR_766.ABI54475.1.Uganda/291-788 Flavivirus envelope glycoprotein E.
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYL
DKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDENRAKVEVT
PNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAK
RQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAG
TDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRG
AKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTA
VSA

SEQ ID NO: 70
5′-(dIdC)$_{13}$-3′
dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC dIdC

SEQ ID NO: 71
KLK peptide
KLKLLLLLKLK

SEQ ID NO: 72
ZIKV Sequence H/PF/2013 as sequenced

CAGACTGCGACAGTTCGAGTTTGAAGCGAAAGCTAGCAACAGTATCAACAGGTTTTATTTTGGATTTGGAAACGAGAGTT
TCTGGTCATGAAAAACCCAAAAAAGAAATCCGGAGGATTCCGGATTGTCAATATGCTAAAACGCGGAGTAGCCCGTGTG
AGCCCCTTTGGGGGCTTGAAGAGGCTGCCAGCCGGACTTCTGCTGGGTCATGGGCCCATCAGGATGGTCTTGGCGATTCT
AGCCTTTTTGAGATTCACGGCAATCAAGCCATCACTGGGTCTCATCAATAGATGGGGTTCAGTGGGGAAAAAAGAGGCTA
TGGAAATAATAAAGAAGTTCAAGAAAGATCTGGCTGCCATGCTGAGAATAATCAATGCTAGGAAGGAGAAGAAGAGAC
GAGGCGCAGATACTAGTGTCGGAATTGTTGGCCTCCTGCTGACCACAGCTATGGCAGCGGAGGTCACTAGACGTGGGAG
TGCATACTATATGTACTTGGACAGAAACGACGCTGGGGAGGCCATATCTTTTCCAACCACATTGGGGATGAATAAGTGTT
ATATACAGATCATGGATCTTGGACACATGTGTGATGCCACCATGAGCTATGAATGCCCTATGCTGGATGAGGGGGTGGAA
CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCACAAAAAAGGTGAAGC
ACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCTGCAAACGCGGTCGCAAACCTGGTTGGAAT
CAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATTGGATATTCAGGAACCCTGGCTTCGCGTTAGCAGCAGCTGCC
ATCGCTTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGC
ATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAAC
ATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGACTGTCGACATAGAGCTGGTTACAACAACAGTCAGCAACAT
GGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCGGACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAA
GCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTG
GACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCC
AGAGAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGACACAGGACATG
AAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCCGAAGCCACCCTGGGGGGTTTTGGAAG
CCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTTTCAGATTTGTATTACTTGACTATGAATAACAAGCACTGGTT
GGTTCACAAGGAGTGGTTCCACGACATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAACTCCACACTGGAACAACA
AAGAAGCACTGGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAGCAG
TTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCCTCTGGCCACTTGAAATG
TCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTGTGTACCGCAGCGTTCACATTCACCAAGATCCC
GGCTGAAACACTGCACGGGACAGTCACAGTGGAGGTACAGTACGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAG
ATGGCGGTGGACATGCAAACTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAA
CTCTAAGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGGGAGAAGAAGATCACCC
ACCACTGGCACAGGAGTGGCAGCACCATTGGAAAAGCATTTGAAGCCACTGTGAGAGGTGCCAAGAGAATGGCAGTCTT
GGGAGACACAGCCTGGGACTTTGGATCAGTTGGAGGCGCTCTCAACTCATTGGGCAAGGGCATCCATCAAATTTTTGGAG
CAGCTTTCAAATCATTGTTTGGAGGAATGTCCTGGTTCTCACAAATTCTCATTGGAACGTTGCTGATGTGGTTGGGTCTGA
ACACAAAGAATGGATCTATTTCCCTTATGTGCTTGGCCTTAGGGGGAGTGTTGATCTTCTTATCCACAGCTGTCTCTGCTGA
TGTGGGGTGCTCGGTGGACTTCTCAAAGAAGGAGACGAGATGCGGTACAGGGGTGTTCGTCTATAACGACGTTGAAGCC
TGGAGGGACAGGTACAAGTACCATCCTGACTCCCCCCGTAGATTGGCAGCAGCAGTCAAGCAAGCCTGGGAAGATGGTA
TCTGTGGGATCTCCTCTGTTTCAAGAATGGAAAACATCATGTGGAGATCAGTAGAAGGGGAGCTCAACGCAATCCTGGAA
GAGAATGGAGTTCAACTGACGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGC
CTGTGAACGAGCTGCCCCACGGCTGGAAGGCTTGGGGGAAATCGTACTTCGTCAGAGCAGCAAAGACAAATAACAGCTT
TGTCGTGGATGGTGACACACTGAAGGAATGCCCACTCAAACATAGAGCATGGAACAGCTTTCTTGTGGAGGATCATGGGT
TCGGGGTATTTCACACTAGTGTCTGGCTCAAGGTTAGAGAAGATTATTCATTAGAGTGTGATCCAGCCGTTATTGGAACAG
CTGTTAAGGGAAAGGAGGCTGTACACAGTGATCTAGGCTACTGGATTGAGAGTGAGAAGAATGACACATGGAGGCTGA
AGAGGGCCCATCTGATCGAGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGACAGATGGAATAGAAGAGAG
TGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCTACAGGACCCAAATGAAAG
GGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCACGTGGAGGAAACATGTGG
AACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGATCGAGGAATGGTGCTGCAGGGAGTGCACAAT
GCCCCCACTGTCGTTCCGGGCTAAAGATGGCTGTTGGTATGGAATGGAGATAAGGCCCAGGAAAGAACCAGAAAGTAAC
TTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGTGCTTGTGATTCTGCTCATG
GTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCGATGGCAGTGCTGGTAGCTATGATCC
TGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTTGATGGGTGCCACCTTCGCGGAAATGAACACTGGAGGA
GATGTAGCTCATCTGGCGCTGATAGCGGCATTCAAAGTCAGACCAGCGTTGCTGGTATCTTTCATCTTCAGAGCTAATTGG
ACACCCCGTGAAAGCATGCTGCTGGCCTTGGCCTCGTGTCTTTTGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGATG
GTTCTCATCAATGGTTTTGCTTTGGCCTGGTTGGCAATACGAGCGATGGTTGTTCCACGCACTGATAACATCACCTTGGCA
ATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGGAGAGCAGGCCTTGCTACTTGCGGGGGGTT
TATGCTCCTCTCTCTGAAGGGAAAAGGCAGTGTGAAGAAGAACTTACCATTTGTCATGGCCCTGGGACTAACCGCTGTGA

GGCTGGTCGACCCCATCAACGTGGTGGGACTGCTGTTGCTCACAAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGT
ACTCACAGCTGTTGGCCTGATATGCGCATTGGCTGGAGGGTTCGCCAAGGCAGATATAGAGATGGCTGGGCCCATGGCC
GCGGTCGGTCTGCTAATTGTCAGTTACGTGGTCTCAGGAAAGAGTGTGGACATGTACATTGAAAGAGCAGGTGACATCAC
ATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCGATGTGGCGCTAGATGAGAGTGGTGATTTCTCCCTG
GTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAGGTGGTCCTGATGACCATCTGTGGCATGAACCCAATAGC
CATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACTGGAAAAAGGAGTGGTGCTCTATGGGATGTGCCTGCTC
CCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAGACTGCTAGGTTCAACACAAGT
TGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCACAAAAGGATCCGCGCTGAGAAGCGGTGAA
GGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCT
GGGACGGGCACAGCGAGGTGCAGCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAA
TATTTAAGACAAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAACTTCAGGATCTCCAATCCTAGAC
AAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGTTATGTTAGTGCCATCACCCAAG
GGAGGAGGGAGGAAGAGACTCCTGTTGAGTGCTTCGAGCCTTCGATGCTGAAGAAGAAGCAGCTAACTGTCTTAGACTT
GCATCCTGGAGCTGGGAAAACCAGGAGAGTTCTTCCTGAAATAGTCCGTGAAGCCATAAAAACAAGACTCCGTACTGTGA
TCTTAGCTCCAACCAGGGTTGTCGCTGCTGAAATGGAGGAAGCCCTTAGAGGGCTTCCAGTGCGTTATATGACAACAGCA
GTCAATGTCACCCACTCTGGAACAGAAATCGTCGACTTAATGTGCCATGCCACCTTCACTTCACGTCTACTACAGCCAATCA
GAGTCCCCAACTATAATCTGTATATTATGGATGAGGCCCACTTCACAGATCCCTCAAGTATAGCAGCAAGAGGATACATTT
CAACAAGGGTTGAGATGGGCGAGGCGGCTGCCATCTTCATGACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGA
CTCCAACTCACCAATTATGGACACCGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATC
ATTCTGGAAAAACAGTTTGGTTTGTTCCAAGCGTGAGGAACGGCAATGAGATCGCAGCTTGTCTGACAAAGGCTGGAAA
ACGGGTCATACAGCTCAGCAGAAAGACTTTTGAGACAGAGTTCCAGAAAACAAAACATCAAGAGTGGGACTTTGTCGTG
ACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGACCGTGTCATAGATTCCAGGAGATGCCTAAAGCCGGTCAT
ACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGTCACACATGCCAGCGCTGCCCAGAGGAGGGGGCGCATA
GGCAGGAATCCCAACAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGACTGACGAAGACCATGCACACT
GGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGATGGCCTCATAGCCTCGCTCTATCGACCTGAGGCCGACA
AAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACCTTTGTGGAACTCATGAAAAGAGGAG
ATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAATAACCTACACAGATAGAAGATGGTGCTTTGATGGCACGA
CCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTGTGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGA
GGTGGATGGACGCCAGAGTTTGTTCAGATCATGCGGCCCTGAAGTCATTCAAGGAGTTTGCCGCTGGGAAAAGAGGAGC
GGCTTTTGGAGTGATGGAAGCCCTGGGAACACTGCCAGGACACATGACAGAGAGATTCCAGGAAGCCATTGACAACCTC
GCTGTGCTCATGCGGGCAGAGACTGGAAGCAGGCCTTACAAAGCCGCGGCGGCCCAATTGCCGGAGACCCTAGAGACCA
TTATGCTTTTGGGGTTGCTGGGAACAGTCTCGCTGGGAATCTTTTTCGTCTTGATGAGGAACAAGGGCATAGGGAAGATG
GGCTTTGGAATGGTGACTCTTGGGGCCAGCGCATGGCTCATGTGGCTCTCGGAAATTGAGCCAGCCAGAATTGCATGTGT
CCTCATTGTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAGCCAGAAAAGCAAAGATCTCCCCAGGACAACCAAATGGC
AATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTACCGCCAATGAACTCGGATGGTTGGAGAGAACAAAGAGTG
ACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAACCATAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTC
AGCTTGGGCCATCTATGCTGCCTTGACAACTTTCATTACCCCAGCCGTCCAACATGCAGTGACCACTTCATACAACAACTAC
TCCTTAATGGCGATGGCCACGCAAGCTGGAGTGTTGTTTGGTATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTGG
AGTCCCGCTGCTAATGATAGGTTGCTACTCACAATTAACACCCCTGACCCTAATAGTGGCCATCATTTTGCTCGTGGCGCAC
TACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGCGTGCTGCCCAGAAGAGAACGGCAGCTGGCATCATGAAGA
ACCCTGTTGTGGATGGAATAGTGGTGACTGACATTGACACAATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACA
GGTGCTACTCATAGCAGTAGCCGTCTCCAGCGCCATACTGTCGCGGACCGCCTGGGGGTGGGGGGAGGCTGGGGCCCTG
ATCACAGCGGCAACTTCCACTTTGTGGGAAGGCTCTCCGAACAAGTACTGGAACTCCTCTACAGCCACTTCACTGTGTAAC
ATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTACACAGTAACAAGAAACGCTGGCTTGGTCAAGAGACGTGG
GGGTGGAACAGGAGAGACCCTGGGAGAGAAATGGAAGGCCCGCTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTAC
AAAAAGTCAGGCATCACCGAGGTGTGCAGAGAAGAGGCCCGCCGCGCCCTCAAGGACGGTGTGGCAACGGGAGGCCAT
GCTGTGTCCCGAGGAAGTGCAAAGCTGAGATGGTTGGTGGAGCGGGGATACCTGCAGCCCTATGGAAAGGTCATTGATC
TTGGATGTGGCAGAGGGGGCTGGAGTTACTACGCCGCCACCATCCGCAAAGTTCAAGAAGTGAAAGGATACACAAAAGG
AGGCCCTGGTCATGAAGAACCCATGTTGGTGCAAAGCTATGGGTGGAACATAGTCCGTCTTAAGAGTGGGGTGGACGTC
TTTCATATGGCGGCTGAGCCGTGTGACACGTTGCTGTGTGACATAGGTGAGTCATCATCTAGTCCTGAAGTGGAAGAAGC
ACGGACGCTCAGAGTCCTCTCCATGGTGGGGGATTGGCTTGAAAAAAGACCAGGAGCCTTTTGTATAAAAGTGTTGTGCC
CATACACCAGCACTATGATGGAAACCCTGGAGCGACTGCAGCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCACTCTC
CCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCGAAAAGCAACACCATAAAAAGTGTGTCCACCACGAGCCAGC
TCCTCTTGGGGCGCATGGACGGGCCCAGGAGGCCAGTGAAATATGAGGAGGATGTGAATCTCGGCTCTGGCACGCGGG
CTGTGGTAAGCTGCGCTGAAGCTCCCAACATGAAGATCATTGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGA

AACGTGGTTCTTTGACGAGAACCACCCATATAGGACATGGGCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAG
CGTCCTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAAACCCTGGGATGTGGTGACTGGAGTCACAGGAATAGCCATG
ACCGACACCACACCGTATGGTCAGCAAAGAGTTTTCAAGGAAAAAGTGGACACTAGGGTGCCAGACCCCCAAGAAGGCA
CTCGTCAGGTTATGAGCATGGTCTCTTCCTGGTTGTGGAAAGAGCTAGGCAAACACAAACGGCCACGAGTCTGTACCAAA
GAAGAGTTCATCAACAAGGTTCGTAGCAATGCAGCATTAGGGGCAATATTTGAAGAGGAAAAAGAGTGGAAGACTGCAG
TGGAAGCTGTGAACGATCCAAGGTTCTGGGCTCTAGTGGACAAGGAAAGAGAGCACCACCTGAGAGGAGAGTGCCAGA
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GGTATATGTGGCTAGGGGCTAGATTTCTAGAGTTCGAAGCCCTTGGATTCTTGAACGAGGATCACTGGATGGGGAGAGA
GAACTCAGGAGGTGGTGTTGAAGGGCTGGGATTACAAAGACTCGGATATGTCCTAGAAGAGATGAGTCGCATACCAGGA
GGAAGGATGTATGCAGATGACACTGCTGGCTGGGACACCCGCATCAGCAGGTTTGATCTGGAGAATGAAGCTCTAATCA
CCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCATTGGCCATAATCAAGTACACATACCAAAACAAAGTGGTAAAGGT
CCTTAGACCAGCTGAAAAAGGGAAGACAGTTATGGACATTATTTCGAGACAAGACCAAAGGGGGAGCGGACAAGTTGTC
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GACTTGTGGCTGCTGCGGAGGTCAGAGAAAGTGACCAACTGGTTGCAGAGCAACGGATGGGATAGGCTCAAACGAATG
GCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATTGATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGATATGGG
AAAAGTTAGGAAGGACACACAAGAGTGGAAACCCTCAACTGGATGGGACAACTGGGAAGAAGTTCCGTTTTGCTCCCAC
CACTTCAACAAGCTCCATCTCAAGGACGGGAGGTCCATTGTGGTTCCCTGCCGCCACCAAGATGAACTGATTGGCCGGGC
CCGCGTCTCTCCAGGGGCGGGATGGAGCATCCGGGAGACTGCTTGCCTAGCAAAATCATATGCGCAAATGTGGCAGCTC
CTTTATTTCCACAGAAGGGACCTCCGACTGATGGCCAATGCCATTTGTTCATCTGTGCCAGTTGACTGGGTTCCAACTGGG
AGAACTACCTGGTCAATCCATGGAAAGGGAGAATGGATGACCACTGAAGACATGCTTGTGGTGTGGAACAGAGTGTGGA
TTGAGGAGAACGACCACATGGAAGACAAGACCCCAGTTACGAAATGGACAGACATTCCCTATTTGGGAAAAAGGGAAGA
CTTGTGGTGTGGATCTCTCATAGGGCACAGACCGCGCACCACCTGGGCTGAGAACATTAAAAACACAGTCAACATGGTGC
GCAGGATCATAGGTGATGAAGAAAAGTACATGGACTACCTATCCACCCAAGTTCGCTACTTGGGTGAAGAAGGGTCTACA
CCTGGAGTGCTGTAAGCACCAATCTTAGTGTTGTCAGGCCTGCTAGTCAGCCACAGCTTGGGGAAAGCTGTGCAGCCTGT
GACCCCCCCAGGAGAAGCTGGGAAACCAAGCCTATAGTCAGGCCGAGAACGCCATGGCACGGAAGAAGCCATGCTGCCT
GTGAGCCCCTCAGAGGACACTGAGTCAAAAAACCCCACGCGCTTGGAGGCGCAGGATGGGAAAAGAAGGTGGCGACCT
TCCCCACCCTTCAATCTGGGGCCTGAACTGGAGATCAGCTGTGGATCTCCAGAAGAGGGACTAGTGGTTAGAGGAGACCC
CCCGGAAAACGCAAAACAGCATATTGACGCTGGGAAAGACCAGAGACTCCATGAGTTTCCACCACGCTGGCCGCCAGGC
ACAGATCGCCGAATAGCGGCGGCCGGTGTGGGG

SEQ ID NO: 73

AHZ13508.1, Zika virus polyprotein from Polynesian outbreak (H/PF/2013)

MKNPKKKSGGFRIVNMLKRGVARVSPFGGLKRLPAGLLLGHGPIRMVLAILAFLRFTAIKPSLGLINRWGSVGKKEAMEIIKKFKK
DLAAMLRIINARKEKKRRGADTSVGIVGLLLTTAMAAEVTRRGSAYYMYLDRNDAGEAISFPTTLGMNKCYIQIMDLGHMCDA
TMSYECPMLDEGVEPDDVDCWCNTTSTWVVYGTCHHKKGEARRSRRAVTLPSHSTRKLQTRSQTWLESREYTKHLIRVENWI
FRNPGFALAAAAIAWLLGSSTSQKVIYLVMILLIAPAYSIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIEL
VTTTVSNMAEVRSYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTG
KSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKH
WLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLK
CRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKM
MLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLF
GGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSADVGCSVDFSKKETRCGTGVFVYNDVEAWRDRYKYHP
DSPRRLAAAVKQAWEDGICGISSVSRMENIMWRSVEGELNAILEENGVQLTVVVGSVKNPMWRGPQRLPVPVNELPHGWK
AWGKSYFVRAAKTNNSFVVDGDTLKECPLKHRAWNSFLVEDHGFGVFHTSVWLKVREDYSLECDPAVIGTAVKGKEAVHSDL
GYWIESEKNDTWRLKRAHLIEMKTCEWPKSHTLWTDGIEESDLIIPKSLAGPLSHHNTREGYRTQMKGPWHSEELEIRFEECPG
TKVHVEETCGTRGPSLRSTTASGRVIEEWCCRECTMPPLSFRAKDGCWYGMEIRPRKEPESNLVRSMVTAGSTDHMDHFSLG
VLVILLMVQEGLKKRMTTKIIISTSMAVLVAMILGGFSMSDLAKLAILMGATFAEMNTGGDVAHLALIAAFKVRPALLVSFIFRA
NWTPRESMLLALASCLLQTAISALEGDLMVLINGFALAWLAIRAMVVPRTDNITLAILAALTPLARGTLLVAWRAGLATCGGFM
LLSLKGKGSVKKNLPFVMALGLTAVRLVDPINVVGLLLLTRSGKRSWPPSEVLTAVGLICALAGGFAKADIEMAGPMAAVGLLIV
SYVVSGKSVDMYIERAGDITWEKDAEVTGNSPRLDVALDESGDFSLVEDDGPPMREIILKVVLMTICGMNPIAIPFAAGAWYVY
VKTGKRSGALWDVPAPKEVKKGETTDGVYRVMTRRLLGSTQVGVGVMQEGVFHTMWHVTKGSALRSGEGRLDPYWGDVK
QDLVSYCGPWKLDAAWDGHSEVQLLAVPPGERARNIQTLPGIFKTKDGDIGAVALDYPAGTSGSPILDKCGRVIGLYGNGVVIK
NGSYVSAITQGRREEETPVECFEPSMLKKKQLTVLDLHPGAGKTRRVLPEIVREAIKTRLRTVILAPTRVVAAEMEEALRGLPVRY
MTTAVNVTHSGTEIVDLMCHATFTSRLLQPIRVPNYNLYIMDEAHFTDPSSIAARGYISTRVEMGEAAAIFMTATPPGTRDAFP
DSNSPIMDTEVEVPERAWSSGFDWVTDHSGKTVWFVPSVRNGNEIAACLTKAGKRVIQLSRKTFETEFQKTKHQEWDFVVTT

DISEMGANFKADRVIDSRRCLKPVILDGERVILAGPMPVTHASAAQRRGRIGRNPNKPGDEYLYGGGCAETDEDHAHWLEAR
MLLDNIYLQDGLIASLYRPEADKVAAIEGEFKLRTEQRKTFVELMKRGDLPVWLAYQVASAGITYTDRRWCFDGTTNNTIMEDS
VPAEVWTRHGEKRVLKPRWMDARVCSDHAALKSFKEFAAGKRGAAFGVMEALGTLPGHMTERFQEAIDNLAVLMRAETGS
RPYKAAAAQLPETLETIMLLGLLGTVSLGIFFVLMRNKGIGKMGFGMVTLGASAWLMWLSEIEPARIACVLIVVFLLLVVLIPEPE
KQRSPQDNQMAIIIMVAVGLLGLITANELGWLERTKSDLSHLMGRREEGATIGFSMDIDLRPASAWAIYAALTTFITPAVQHAV
TTSYNNYSLMAMATQAGVLFGMGKGMPFYAWDFGVPLLMIGCYSQLTPLTLIVAIILLVAHYMYLIPGLQAAAARAAQKRTAA
GIMKNPVVDGIVVTDIDTMTIDPQVEKKMGQVLLIAVAVSSAILSRTAWGWGEAGALITAATSTLWEGSPNKYWNSSTATSLC
NIFRGSYLAGASLIYTVTRNAGLVKRRGGGTGETLGEKWKARLNQMSALEFYSYKKSGITEVCREEARRALKDGVATGGHAVSR
GSAKLRWLVERGYLQPYGKVIDLGCGRGGWSYYAATIRKVQEVKGYTKGGPGHEEPMLVQSYGWNIVRLKSGVDVFHMAAE
PCDTLLCDIGESSSSPEVEEARTLRVLSMVGDWLEKRPGAFCIKVLCPYTSTMMETLERLQRRYGGGLVRVPLSRNSTHEMYWV
SGAKSNTIKSVSTTSQLLLGRMDGPRRPVKYEEDVNLGSGTRAVVSCAEAPNMKIIGNRIERIRSEHAETWFFDENHPYRTWAY
HGSYEAPTQGSASSLINGVVRLLSKPWDVVTGVTGIAMTDTTPYGQQRVFKEKVDTRVPDPQEGTRQVMSMVSSWLWKELG
KHKRPRVCTKEEFINKVRSNAALGAIFEEEKEWKTAVEAVNDPRFWALVDKEREHHLRGECQSCVYNMMGKREKKQGEFGKA
KGSRAIWYMWLGARFLEFEALGFLNEDHWMGRENSGGGVEGLGLQRLGYVLEEMSRIPGGRMYADDTAGWDTRISRFDLE
NEALITNQMEKGHRALALAIIKYTYQNKVVKVLRPAEKGKTVMDIISRQDQRGSGQVVTYALNTFTNLVVQLIRNMEAEEVLEM
QDLWLLRRSEKVTNWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRFLNDMGKVRKDTQEWKPSTGWDNWEEVPFC
SHHFNKLHLKDGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQMWQLLYFHRRDLRLMANAICSSVPVDWVPTG
RTTWSIHGKGEWMTTEDMLVVWNRVWIEENDHMEDKTPVTKWTDIPYLGKREDLWCGSLIGHRPRTTWAENIKNTVNMV
RRIIGDEEKYMDYLSTQVRYLGEEGSTPGVL

SEQ ID NO: 74
9320_Zika_PF_1F
ttaggatccGTTGTTGATCTGTGTGAAT

SEQ ID NO: 75
9321_Zika_PF_1R
taactcgagCGTACACAACCCAAGTT

SEQ ID NO: 76
9322_Zika_PF_2F
ttaggatccTCACTAGACGTGGGAGTG

SEQ ID NO: 77
9323_Zika_PF_2R
taactcgagAAGCCATGTCYGATATTGAT

SEQ ID NO: 78
9324_Zika_PF_3F
ttaggatccGCATACAGCATCAGGTG

SEQ ID NO: 79
9325_Zika_PF_3R
taactcgagTGTGGAGTTCCGGTGTCT

SEQ ID NO: 80
9326_Zika_PF_4F
ttaggatccGAATAGAGCGAARGTTGAGATA

SEQ ID NO: 81
9327_Zika_PF_4R
taactcgAGTGGTGGGTGATCTTCTTCT

SEQ ID NO: 82
9328_Zika_PF_5F
ttaggatcCAGTCACAGTGGAGGTACAGTAC

70

SEQ ID NO: 83
9329_Zika_PF_5R
taactcgagCRCAGATACCATCTTCCC

SEQ ID NO: 84
9330_Zika_PF_6F
ttaggatCCCTTATGTGCTTGGCCTTAG

SEQ ID NO: 85
9331_Zika_PF_6R
taactcgagTCTTCAGCCTCCATGTG

SEQ ID NO: 86
9332_Zika_PF_7F
ttaggatccAATGCCCACTCAAACATAGA

SEQ ID NO: 87
9333_Zika_PF_7R
taactcgagTCATTCTCTTCTTCAGCCCTT

SEQ ID NO: 88
9334_Zika_PF_8F
ttaggatccAAGGGTGATCGAGGAAT

SEQ ID NO: 89
9335_Zika_PF_8R
taactcgagTTCCCTTCAGAGAGAGGAGC

SEQ ID NO: 90
9336_Zika_PF_9F
ttaggatccTCTTTTGCAAACTGCGATC

SEQ ID NO: 91
9337_Zika_PF_9R
taactcgagTCCAGCTGCAAAGGGTAT

SEQ ID NO: 92
9338_Zika_PF_10F
ttaggatccGTGTGGACATGTACATTGA

SEQ ID NO: 93
9339_Zika_PF_10R
taactcgagCCCATTGCCATAAAGTC

SEQ ID NO: 94
9340_Zika_PF_11F
ttaggatccTCATACTGTGGTCCATGGA

SEQ ID NO: 95
9341_Zika_PF_11R
taactcgagGCCCATCTCAACCCTTG

SEQ ID NO: 96
9342_Zika_PF_12F

ttaggatccTAGAGGGCTTCCAGTGC

SEQ ID NO: 97
9343_Zika_PF_12R
taactcgAGATACTCATCTCCAGGTTTGTTG

SEQ ID NO: 98
9344_Zika_PF_13F
ttaggatccGAAAACAAAACATCAAGAGTG

SEQ ID NO: 99
9345_Zika_PF_13R
taactcgagGAATCTCTCTGTCATGTGTCCT

SEQ ID NO: 100
9346_Zika_PF_14F
ttaggatccTTGATGGCACGACCAAC

SEQ ID NO: 101
9347_Zika_PF_14R
ttaggatccGTTGTTGATCTGTGTGAAT

SEQ ID NO: 102
9348_Zika_PF_15F
taactcgagCAGGTCAATGTCCATTG

SEQ ID NO: 103
9349_Zika_PF_15R
ttaggatccTGTTGTGTTCCTATTGCTGGT

SEQ ID NO: 104
9350_Zika_PF_16F
taactcgaGTGATCAGRGCCCCAGC

SEQ ID NO: 105
9351_Zika_PF_16R
ttaggatccTGCTGCCCAGAAGAGAA

SEQ ID NO: 106
9352_Zika_PF_17F
taactcgaGCACCAACAYGGGTTCTT

SEQ ID NO: 107
9353_Zika_PF_17R
ttaggatcCTCAAGGACGGTGTGGC

SEQ ID NO: 108
9354_Zika_PF_18F
taactcgagCAATGATCTTCATGTTGGG

SEQ ID NO: 109
9355_Zika_PF_18R
ttaggatccTATGGGGGAGGACTGGT

SEQ ID NO: 110

9356_Zika_PF_19F
taactcGAGCCCAGAACCTTGGATC

SEQ ID NO: 111
9357_Zika_PF_19R
ttaggatcCAGACCCCCAAGAAGGC

SEQ ID NO: 112
9358_Zika_PF_20F
taactcgagCCCCTTTGGTCTTGTCT

SEQ ID NO: 113
9359_Zika_PF_20R
ttaggatccAGGAAGGATGTATGCAGATG

SEQ ID NO: 114
9360_Zika_PF_21F
taactcgagACATTTGCGCATATGATTTTG

SEQ ID NO: 115
9361_Zika_PF_21R
ttaggatccAGGAAGGACACACAAGAGT

SEQ ID NO: 116
9362_Zika_PF_22F
taactcgagACAGGCTGCACAGCTTT

SEQ ID NO: 117
9363_Zika_PF_22R
ttaggatccTCTCTCATAGGGCACAGAC

In some embodiments, the Zika virus has polyprotein, including an envelope (E) protein, with an amino acid sequence provided by any one of SEQ ID NOs: 14-69 or 72. In some embodiments, the polyprotein or E protein sequence is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%. 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to any one of SEQ ID NOs: 2-69 or 72.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or amino acid sequences refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity) over a specified region or over the entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more

amino acids) in length. In some embodiments, the identity exists over the length of a protein, such as the E protein.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.,* by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. MoI. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman. Proc. Natl. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, Jalview and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. 575 Science Dr., Madison. WI), by multi sequence alignment implementation using e.g. CLUSTALW (Larkin et al., (2007). Bioinformatics, 23, 2947-2948.) or MAFFT (Katoh & Toh 2008 Briefings in Bioinformatics 9:286-298), or by manual alignment and visual inspection (see. *e.g.,* Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1977 and Altschul et al., J. MoI. Biol. 215:403-410, 1990, respectively.

EXAMPLES

Example 1: Production of a Zika drug substance suitable for application as a vaccine in humans and animals

Materials and Methods:

For the production of ZikaV the JEV process platform (Srivastava et al., Vaccine 19 (2001) 4557-4565; US 6,309,650B1) was used as a basis. Small changes of certain process steps were adapted to ZikaV properties and to improve purity. A short summary of the process steps is outlined below (see also Figure 9A and B). Briefly, the unexpected and novel purification properties of protamine sulphate (PS) were evaluated in purification processes for Zika Virus. As shown in Figure 10, non-infectious virus particle aggregates, HCP and other LMW impurities were removed by PS precipitation as shown by removal of aggregate shoulder in SEC-HPLC and no loss of infectious virus titer by PS treatment. Further optimization of the Zika purification protocol is provided below.

Upstream:

- Roller Bottle based Vero cell expansion (25x850cm2 CellBind):
- 5% CO_2, 35°C, MEM+2mM L-Glutamine + 10% FBS
- Infection with ZikaV research Master Seed Bank (rMSB) at MOI 0.01
- Virus Production without serum
- 5% CO_2, 35°C, MEM+2mM L-Glutamine
- Multiple harvests (days 2, 3, 5 and 7) with re-feed
- Sterile filtration of harvests and storage at 2-8°C until further processing

Downstream:

- Pooling of harvests and concentration by ultrafiltration (100kDa)
- Stabilization of concentrated harvest (Tris/10% sucrose) for storage if required (-80°C)
- Removal of hcDNA by Protamine Sulphate (2 mg/mL)
- Sucrose Gradient Purification (optimized three layered gradient)
- Formaldehyde Inactivation (0.02%, 22°C, 10 days), neutralization with Na-metabisulfite
- Dilution to DS antigen target content and formulation with Aluminium hydroxide (0.5mg Al/mL)

Zika Virus Strain H/PF/2013 was originally isolated from a 51-year-old woman (accession number KJ776791.1, also SEQ ID NO: 13 herein) from French Polynesia. A sample was obtained from the European Virus Archive (EVAg; Ref-SKU: 001v-EVA1545). Based on this material, a research master seed bank (rMSB) was prepared on Vero cells as the cell substrate and the genomic sequence was checked by sequencing. Because the genomic sequence at the 5´and 3´flanking sequences of Zika virus strain H/PF/2013 was unknown, primers for sequencing were designed in those regions based on other Zika virus strains whereas the internal primers were designed from the published sequence (SEQ ID NOs: 74 to 117, see also Table A). The sequence obtained from the rMSB by use of these primers is provided by SEQ ID NO: 72. There was 100% overlap of the sequence with the published sequence of Zika Virus Strain H/PF/2013 (SEQ ID NO: 13). However, we sequenced additional regions 5´ (an additional 40 bp) and 3' (an additional 160 bp) represented in SEQ ID NO: 72. In a preferred embodiment, the Zika virus of the invention comprises SEQ ID NO: 72. The genomic RNA is somewhat longer than the sequence according to SEQ ID NO: 72 (perhaps an additional 200 bp). Additionally, a Zika virus adapted to a host cell such as e.g. Vero cells may be expected to contain one or more mutations. For these reasons, the Zika virus of the current invention comprises the sequence of SEQ ID NO: 72 or, preferably, a sequence with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 72. Furthermore, because the viral genome is likely to contain even further flanking regions to SEQ ID NO: 72; in one embodiment, the Zika virus of the invention contains the sequence of SEQ ID NO: 72 and optionally further comprises

extensions at the 5' and/or 3' ends of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120 or at least 130 nucleotides. In a preferred embodiment, the Zika virus comprises at least the coding sequence for the entire polyprotein of Zika Virus Strain H/PF/2013 of the invention i.e. the amino acid sequence of SEQ ID NO: 73 or a polyprotein with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 73. Furthermore, the Zika virus comprises at least the coding sequence for the E-protein of Zika Virus Strain H/PF/2013 of the invention SEQ ID NO: 47 or an E-protein thereof with at least 95%, 96%, 97%, 98%, or at least 99% sequence identity to the sequence provided by SEQ ID NO: 47.

Virus growth on Vero Cells

Vero cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers were infected with Zika Virus Strain H/PF/2013 at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures were washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures were incubated until the virus titer reaches a desired level.

The culture medium was harvested at days 2, 3, 5 and 7 and were pooled from those harvest days and then centrifuged in a standard centrifuge. The supernatants were then filtered. Virus culture supernatants were concentrated by TFF ultrafiltration to remove cell culture media components and to reduce batch volume.

Evaluation of harvest Procedure

The current JEV harvest process has scheduled harvests on days 3, 5, 7 and 9 post infection. To mimic the JEV process roller bottles were infected with ZIKV bank P4-FBS at an MOI of 0.01 in infection medium (MEM with 2% FBS + 2mM L-glutamine) for 2 hours. After removing the inoculum the cells were washed twice with PBS and 200 mL production medium (MEM+ 2mM L-glutamine) was added.

After taking a sample on day 2 the first virus harvest was conducted on day 3 after infection. At this point significantly higher CPE could be observed compared to cells where virus was removed on day 2. Plaque assay analysis showed that the viral titers on day 2 were in the same range as for the standard harvesting schedule. However, starting with the day 3 harvest, the observed titers were significantly lower correlating with the increased CPE observed compared to the standard harvest

schedule. On day 5 post infection no more living cells could be observed at all and the experiment was terminated with a final day 5 harvest.

Table 3: The calculated titers per plaque assay are summarized in the list below.

	Log 10 PFU/mL
sample day2	7.02
harvest day 3	6.66
harvest day 5	6.26

This finding led to an optimized harvest schedule to better control of CPE and allow additional harvest day 5 and 7, see Figure 15. For both harvest days the optimized ZikaV protocol yield significant higher virus titers compared to the modified protocol showing that the time of the first harvest is crucial for production yields. Additionally first harvesting at day 3 results in maximum 2 harvest points whereas first harvesting at day 2 allows for 4 harvest points further increasing the yield gain.

Downstream Purification of Zika virus

The purification process was carried out at room temperature (18-22°C) unless stated otherwise. Virus purification started with concentration of filtered combined harvest using 100 kDa cut-off TFF ultrafiltration modules to remove cell culture media components and reduce batch volume. After concentration, the pooled filtered harvest material was adjusted to a final concentration of 25 mM Tris pH 7.5 and 10% sucrose (w/w) using stock solution of both components (see Figure 11 for SEC-HPLC of different harvests prior to PS treatment). This allowed for freezing the concentrated harvest at <-65°C if required.

Host cell DNA and protein reduction as well reduction of non-infectious virus aggregates in the concentrated material was achieved by precipitation with protamine sulphate (2 mg/mL) followed by sucrose density centrifugation (2-8°C) as final polishing step (see Figure 20 for SEC-HPLC of different harvests post PS treatment). The purification process was designed to be completed within 2 working days with SGC starting on end of day 1 followed by fractionation and SDS-PAGE analysis on day 2. The sucrose gradient fractions were stored at 2-8 °C during the SDS-PAGE analysis (Silver staining) to identify the pure fractions containing ZikaV (see Figure 21). After pooling the relevant fractions, the pool was diluted and inactivated by Formalin. After pooling the relevant fractions of sucrose gradient centrifugation, the pool was diluted 1:3 in PBS and inactivated by Formalin (0.02% v/v, 200ppm). Fractions were subjected to analysis by SDS-PAGE.

Effect of PS treatment on virus recovery

Samples of individual 30x concentrated harvests days 2, 3, 5 and 7 were analysed before (Figure 11) and after PS (Figure 12) treatment by SEC-HPLC and plaque assay. SEC-HPLC was used for determination of relative total ZikaV content (active + inactive) expressed as peak area, whereas the rel. ZikaV peak purity is given as relative content of virus monomer population to total virus peak. Plaque assay states the content of total active virus particles in each sample. Experimental results are summarized in Table 4. The virus peak recovery by SEC-HPLC was only between 12 to 36% with peak purity after PS treatment in the range of >90% (no virus aggregates detected). The recovery of active virus particles by plaque assay was all > 100% (130-700%, range within the variability of the assay) showing that no active virus particles were lost during PS treatment. These results show that during PS treatment only non-infective (immature and/or aggregated virus) particles were removed.

Table 4: ZikaV recovery by SEC-HPLC and plaque assay before and after PS treatment.

SEC-HPLC

	Peak area mAU*min			
Harvest day	30x conc	30x + PS	SEC Recovery (%)	rel. virus monomer content after PS (%)
Day 2	101.36	18.63	18	89%
Day 3	144.51	17.48	12	90%
Day 5	19.97	5.92	30	96%
Day 7	68.80	24.43	36	99%

Plaque Assay

	PFU/mL		
Harvest day	30x conc	30x + PS	Plaque Recovery (%)
Day 2	3E+08	5E+08	179
Day 3	2E+08	4E+08	193
Day 5	1E+08	9E+08	700
Day 7	3E+08	4E+08	132

Sucrose gradient centrifugation

The PS treated harvest was split in two parts and loaded on two centrifuge bottles. Sucrose density gradient centrifugation (SGC) was used for final concentration and polishing of the ZikaV material. The ZikaV PS treated concentrated harvest was loaded on top of a solution consisting of three layers of sucrose with different densities. The three sucrose layers were selected based on a preliminary study which showed the formation of a linear sucrose gradient and complete separation of the virus

particles from residual contaminants as demonstrated for ChikV (Fig. 15D). The optimal volumes of the sucrose solutions were determined empirically. The volumes of individual layers for a centrifugation in 100 mL bottle scale are shown in Table 5.

Table 5: Individual layers/volumes for a centrifugation in bottle.

Solution	**Volume (mL)**
PS treated harvest in 10% sucrose (L)	40
15% sucrose (J)	15
35% sucrose (I)	15
50% sucrose (H)	20
Total volume	90

The sucrose gradient bottles were prepared by stratifying the individual sucrose layers. A plastic tube was attached to peristaltic pump tubing. The plastic tube was mounted on a laboratory stand using a clamp and placed into the centrifuge bottle. The nozzle of the plastic tube was touching the bottom of the bottle. Using a peristaltic pump the ZikaV material and the sucrose solutions were pumped into the cylinder. A measuring cylinder was used as feed vessel. The first solution pumped was the ZikaV material as it represented the solution of lowest density (10 % sucrose (w/w)). After the ZikaV material the sucrose solutions were pumped in ascending order starting with the 15 % (w/w) solution J, followed by 35 % sucrose solution I and finishing with the highest density sucrose solution H (50 % (w/w)). The described setup is shown in Figure 14. After all sucrose solutions were transferred the plastic tubing was carefully removed in order not to disturb the layers.

Prior to centrifugation the centrifuge was pre-cooled to 4°C. The prepared SG bottles were carefully transferred into the pre-cooled rotor. (Note: Sudden movement of the bottles during transfer to the rotor must be avoided in order not to disturb the sucrose layers.) The bottles were centrifuged at ~11.000 RCF max at 4 °C for at least 20 hours, no brake/deceleration activated. In case a different centrifuge system with a different rotor is used the necessary speed and centrifugation times need to be calculated based on the k-factor in order to achieve comparable centrifugation efficiency.

Harvesting of the sucrose gradient was done manually using a peristaltic pump. A plastic tube attached to peristaltic pump tubing was used for harvesting the sucrose gradient. The bottle containing the gradient was mounted onto a laboratory stand in a tilted position (~12°) using a clamp. The plastic tubing was then placed into the bottle touching the bottom edge of the bottle and was fastened in

position using a clamp. This resulted in a small gap of 1-2 mm between the tubing inlet and the bottom of the bottle (see Figure 14).

Using a peristaltic pump set to a flow rate of 30 mL per minute the gradient was harvested and manually split into 2 mL fractions. A total number of 32 fractions per bottle were harvested (~ 64 mL) and the remaining volume was discarded. The fractions were immediately tested by SDS-PAGE / silver stain to identify the virus containing fractions with sufficient high purity. Representative SDS-PAGE is shown in Figure 14. Fraction 10-14 were pooled and further processed.

The purified viral solution was inactivated by incubation with 0.02% formaldehyde over a period of ten days in a 22°C controlled-temperature incubator. The formaldehyde is neutralized by addition of sodium metabisulphite on the tenth day.

The sucrose gradient pool (~17mL after sampling) was further diluted 3-fold with PBS to a final volume of 51 mL in a PETG container. A volume of 1% formaldehyde (10,000 ppm) solution equivalent to 1/50 of the final volume of the pre-formaldehyde pool was added to this pool resulting in an effective concentration of 200 ppm. The formaldehyde-treated solution was mixed on a magnetic stirrer for 10 minutes. After sampling, the formaldehyde-treated viral solution was placed within a cooled incubator at 22°C ± 2°C. On Day 5 post addition of formaldehyde, the formaldehyde-treated viral solution was filtered through a 0.2 µm filter and then placed in the incubator at 22°C ± 2°C again. On Day 10, after removing the 10-Day inactivation final sample, a volume of 1 % (of the weight of the final formaldehyde-treated viral solution) of 200 mM-sodium metabisulphite solution (2mM final concentration) was aseptically transferred into the PETG container containing the formaldehyde-treated viral solution. After mixing for 5 minutes on a magnetic stirrer, the neutralized inactivated viral solution is held at room temperature (20 to 25°C) for a minimum of 30 minutes. After sampling, the neutralized inactivated viral solution is stored at 5°C ± 3°C until further processing.

Inactivation by formaldehyde

Critical parameters for this step are final formalin concentration, temperature, mixing and transfer into a new container. A preliminary acceptance criterion for maximum pfu/mL (determined by plaque assay) has been set on the diluted pool pre formaldehyde treatment.

The quality of the neutralized inactivated viral solution was monitored by the following parameters: Plaque assay on Day 10, SEC-HPLC, SDS-PAGE/Western Blot.

Interestingly, SEC-HPLC analysis of samples taken during the inactivation period followed by neutralization with bisulfite showed more or less constant peak area throughout the inactivation period. This is in contrast to JEV where losses of viral particles up to 60% are observed using the process disclosed by Srivastava et al. Vaccine 19 (2001) 4557-4565. In a scale-down model the viral losses were even much higher due to surface/area ratio at smaller scale and high losses due to unspecific adsorption. Differences of the ZikaV inactivation experiment and JEV inactivation were noticed as follows:

A) Much higher purity of ZikaV SGP pool with regard to residual PS (<2µg/mL) compared to JEV. The 3-fold ZikaV inactivated sample contained therefore <<1µg/mL of residual PS. Commercial JEV SGP pool contains on average ~120µg/mL (up to 152 µg/mL possible). The average dilution to inactivation solution of ~14-fold results in a residual PS content up to ~11 µg/mL. It may be that higher amount of residual PS could cause virus precipitation due to cross-linking / reaction with formalin.

B) ZikaV inactivation sample contained ~10% sucrose (3-fold dilution of SGP pool containing ~30-35% sucrose). Sucrose might have stabilizing effect of viral ZikaV particles during treatment with formalin.

Dilution to DS and Formulation with Aluminium hydroxide (DP)

For preparation of ZikaV drug substance used in mouse potency assay an antigen content (expressed as total viral particles or SEC peak area) of 5 times higher compared to Ixiaro was targeted. The basis for determination of antigen content was SEC-HPLC. Briefly, a Superose 6 10/300 Increase column (GE Healthcare) equilibrated with PBS + 250 mM NaCl, pH 7.4 at 1 ml/min and 25°C, was used to detect ZikaV at 214 nm detection wavelength in harvest samples and throughout the downstream process. In the current JEV process the antigen content in NIV is determined by a specific ELISA. A good correlation was observed between antigen content determined by ELISA and SEC-HPLC. On average, the antigen content in commercial NIV samples is in the range of 33 AU/mL corresponding to ~5.2mAU JEV peak area, see Figure 21.

ZikaV NIV day10 (Zika peak ~36 mAU, analysed on Waters HPLC/Superose6 Increase column) was diluted with PBS to a target of 6.3 (~5.7x dilution). Aluminium hydroxide was added to a final concentration of 0.5 mg/mL Aluminium (1/20 v/v Alum 2% stock solution added) to prepare ZikaV Drug Product (DP). The DP was gently mixed for 5 min. An aliquot of the DP was removed, Alum sedimented by centrifugation and the clear supernatant analysed by SEC-HPLC. No ZikaV peak was detected in the supernatant indicating complete adsorption (estimated as >95%) of viral particles on the mineral adjuvant. Formulated ZikaV DP was stored at 2-8°C.

The impurity profile of the inactivated Zika virus DS is comparable to the profile of JEV DS with the exception of a lower PS content (Table 6).

Table 6: Determination of impurity profile in Zika and JEV DS samples:

	Specification (JEV DS)	**JEV**	**Zika**
HCP (ng/mL)	<100 LOQ 12 ng/mL	<LOQ	<LOQ
DNA (pg/mL)	<200 LOQ 40 pg/mL	<40	<40
Aggregates by SEC-MALLS (%)	Not specified, part of characterization LOQ 5%	<LOQ	<LOQ
PS (µg/mL)	Specification only at SGP pool to demonstrate consistent process performance (19-152µg/mL), *PS content in DS calculated based on PS content in SGP pool (~100 µg/mL) and average dilution factor (~28x) to DS; LOQ 2 µg/mL	~4*	<<LOQ

*Typical PS impurity in a JEV sample produced in accordance with protocol disclosed in Srivastava et al. Vaccine 19 (2001) 4557-4565.

SEC-MALLS Results

A representative SEC-HPLC elution profile of ZikaV NIV at 214 nm detection wave length is shown in Figure 16. Note that BSA (50µg/mL) was added to the sample to minimize losses in HPLC glass vial due to unspecific surface adsorption. ZikaV monomer content was estimated as ~98% with a multimer content of ~2%.

SEC-MALLS analysis (Figure 17) of the sample confirmed the radius Rz of the monomer ZikaV population peak 1 as 21.6 nm and ~49 nm for the multimer peak 2. Cumulative particle size distribution showed that 89% of all viral particles are within a radius range between 18 to 25nm (Figure 18).

Results confirm purity and homogeneity of ZikaV NIV.

Viral titer by plaque assay

Table 7: Active ZikaV pfus were quantified by plaque assay throughout the process.

Sample	**Pfu/mL**
Harvest day 2 (filtered)	$6.4x10^7$
Harvest day 3 (filtered)	$1.0x10^8$
Harvest day 5 (filtered)	$1.5x10^8$

Harvest day 7 (filtered)	$1.1x10^8$
PS treated harvest 300x concentrate (=SGP load)	$9.0x10^8$
SGP pool	$8.9x10^8$
Inactivation start (SGP pool 1:3 diluted)	$3.4x10^8$
Inactivation day 5	<LOD
Inactivation day 10	<LOD

Comparison of PS and Benzonase on process performance

A direct comparison of DNA removal method of concentrated ZikaV harvest pool was done. One aliquot was treated with PS (2 mg/mL, 15min at room temperature), the other aliquot was treated with Benzonase (50 U/mL, 2 mM MgCl2, 4h RT, 48h 2-8°C). Both samples were further purified by sucrose gradient as described in this report. Interestingly, the Benzonase treated samples did not yield any pure fractions after sucrose gradient centrifugation of the treated ZikaV harvest. In those fractions where the specific virus bands were detected, a high amount of host cell protein was detected throughout the collected fractions. The PS treated material resulted in pure ZikaV containing fractions as expected. This finding may suggest that PS is not only effective for DNA removal by precipitation; in addition it improves the recovery of virus particles in the gradient by disrupting interaction of DNA (fragments) and virus particles. Benzonase treatment does not remove DNA, it only results in its fragmentation. Residual DNA fragments might still interact with virus particles and residual HCPs resulting in cross-contamination and co-purification in the sucrose gradient. Pooled SGP fractions were also analysed by SEC-HPLC. Although a large peak was detected, SDS-PAGE confirmed that this sample was highly contaminated with HCPs. A large peak might be detected at UV214 and 280nm after SEC-HPLC analysis due to possible interaction of HCPs with large virus particles, changing the UV absorbance.

Immunogenicity of Vero grown Zika virus

Immunization of mice

Prior to immunization, groups of ten 6-week-old female CD1 mice were bled via vena facialis and pre-immune sera were prepared. One intraperitoneal immunizations of 200 µL were administered. A dose titration (12 µg, 3 µg, 1 µg, 0.33 µg, 0.11 µg, 0.037 µg and 0.012 µg, equivalent to the protein amount in IXIARO) of inactivated Zika virus formulated with aluminium hydroxide (Al(OH)3) at a final concentration of 0.7%. Three weeks after immunization, blood was collected and immune sera were prepared. All animal experiments were conducted in accordance with Austrian law (BGB1 Nr. 501/1989) and approved by "Magistratsabteilung 58".

Plaque reduction neutralization test (PRNT)

Twelve well plates were used for PRNT. Each well was seeded with 1 mL medium containing $4x10^5$ Vero cells and incubated 35°C with 5% CO_2 overnight. Pools of heat inactivated sera from each dose group were tested in triplicate. The target viruses (H/PF/2013 (SEQ ID NO: 13) or MR766 (SEQ ID NO: 11)) were diluted to 100 pfu/165 µL. Equal volumes of target virus and serum dilution were incubated at 35°C with 5% CO_2 for 1 hour. The cell culture medium was aspirated from the Vero cells and 330 µL of the mixture target virus/serum dilution were added to each well and the plates were rocked back and forth 5 times before incubating for 2 hours at 35°C with 5% CO_2. To each well 1 mL of a 2% methylcellulose solution containing EMEM and nutrients was added, the plates were then incubated for 5 days at 35°C with 5% CO_2 before staining the cells for 1 hour with crystal violet/5% formaldehyde and subsequently washed 3 times with deionized water. The plates were air dried and the numbers of plaques in each well were manually counted.

Results

Neutralization was observed with serum pools from mice immunized with inactivated Zika virus vaccine (H/PF/2013) down to 37 ng (dosing equivalent to the amount protein in IXIARO®) against Zika viruses of both the Asian (H/PF/2013) and African (MR766) lineages (Figures 19 and 20, respectively). Complete inhibition was seen at the 1:20 serum dilution with an immunization dose down to 110 ng (dosing equivalent to the amount protein in IXIARO®). The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

Another neutralization assay was performed using the microneutralization assay as described by Larocca, et al. (2016, Nature doi:10.1038/nature18952). It was found that the inactivated Zika virus of the current invention had an MN50 (microneutralization) titer of 90 at 1 µg of inactivated purified virus.

Further methods: The immunogenicity of inactivated Zika virus preparations is assessed using a mouse model of Zika infection. Groups of adult mice are immunized subcutaneously (s.c.) with 500, 50, or 5 ng of inactivated Zika virus with adjuvant (*e.g.* aluminium hydroxide with or without IC31®), or without adjuvant. An additional group of mice receive PBS as a negative control. Each group is administered the indicated inoculum at t=0 and in some cases also at three to four weeks later (t=3/4). Beginning approximately three weeks after administration of the last immunization, serum samples are obtained from each of the mice at regular intervals. The serum samples are tested for the presence of neutralizing antibodies using PRNT.

The *in vivo* protective efficacy of the inactivated Zika virus preparations is also assessed using a mouse model of Zika infection, *i.e.* IFN-alpha/beta receptor knock-out mice (A129) (see *e.g.* Dowall et al., 4. March 2016, dx.doi.org/10.1101/042358) or blocking of the IFN-alpha/beta receptor by administration of anti-IFN-alpha/beta receptor monoclonal antibodies to C57BL/6 or BALB/c mice (see e.g. Pinto et al., 7. December 2011, DOI: 10.1371/journal.ppat.1002407). For protection assays, groups of 10 three- to eight-weeks-old A129, C57BL/6 of BALB/c mice are inoculated subcutaneously in the hindquarters with inactivated Zika virus with adjuvant (aluminium hydroxide) or without adjuvant at t=0. Age-matched controls are inoculated with PBS or non-specific antigens in alum. Mice are optionally boosted with a second administration of the indicated inoculation three to four weeks later. The mice are then challenged subcutaneously at three to eight weeks post immunization by inoculation with a deadly dose of live Zika virus. One day prior to challenge of C57BL/6 and BALB/c mice, they are passively administered (intraperitoneally) anti-IFN-alpha/beta receptor monoclonal antibodies. Challenged mice are monitored daily for morbidity and mortality for up to twenty-one days. Another alternative is to challenge intracranially adult vaccinated/non-vaccinated adult mice and observe protection.

It is expected that the Zika virus produced by the process of the invention will provide very similar functional read-outs in *in vitro*, *in vivo* and finally human trials as the currently licensed JEV vaccine in the EU and US and elsewhere, IXIARO®. The dosage may alter but due to the very similar impurity profile and almost identical manufacture, a very similar efficacy and safety result will be expected as was determined for the currently licensed JEV vaccine (licensed in the EU and US and elsewhere).

Discussion & Conclusion

The existing manufacturing platform for production of inactivated JEV vaccine IXIARO® was used as a basis for a manufacturing feasibility study of inactivated ZikaV vaccine candidate (Asian strain H/PF/2013). The virus was produced on Vero cells cultivated in roller bottles. The virus was purified by PS treatment followed by an optimized sucrose gradient. Inactivation was done by formalin treat (0.02 %, 10 days at 22 °C). For exploratory immunization studies in mice, a DP formulated with Alum was prepared with an estimated 5-fold higher virus particle content compared to IXIARO®, the commercial JEV Vaccine. The impurity profile of the DS met all criteria as defined in the specification for IXIARO®, the commercial JEV vaccine. The neutralization of both the Asian (H/PF/2013) and African (MR766) lineages of the Zika virus was equivalent, which indicates high

cross-neutralization between different Zika virus strains of the inactivated Zika virus vaccine (H/PF/2013).

The in vivo data regarding immunogenicity of the inactivated Zika virus vaccine of the current invention indicates that the virus is surprisingly potently immunogenic and also highly cross-protective (very similar immunogenicity in African and Asian strains). Data indicate that immunogenicity was higher than the recently reported inactivated Zika virus vaccine candidate (Larocca, et. al, 2016, supra.). Inactivated viruses are among the safest vaccines and especially preferred for deliver to populations where safety is especially concerning, such as pregnant women, children and immunocompromised individuals, which makes the herein disclosed inactivated Zika virus particularly suitable. Obtaining a high titer of inactivated virus is a challenge in the field. The herein disclosed process for purifying inactivated Zika virus results in not only a high yield, but also a very pure drug substance.

Further more detailed aspects of the invention:

A1. A Zika virus vaccine comprising an inactivated Zika virus particle, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus vaccine with at least a 70% probability.

A2. The Zika virus vaccine of A1, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability.

A3. The vaccine of A1 or A2, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13 or 72, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 or 72 and able to pack a virulent Zika virus.

A4. The vaccine of any one of A1-A3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 and able to pack a virulent Zika virus.

A5. The vaccine of any one of A1-A4, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

A6. The vaccine of A5, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent to completely inactivate the Zika virus as measured by plaque assay.

A7. The vaccine of A6, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

A8. The vaccine of A7, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

A9. The vaccine of any one of A5-A8, wherein the chemical activation is performed at about +4°C or about +22°C.

A10. The vaccine of any one of A1-A9, further comprising an adjuvant.

A11. The vaccine of A10, wherein the adjuvant is an aluminum salt adjuvant.

A12. The vaccine of A11, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

A13. The vaccine of any one of A10-A12, wherein the vaccine comprises or further comprises an adjuvant comprising a peptide and a deoxyinosine-containing immunostimulatory oligodeoxynucleic acid molecule (I-ODN).

A14. The vaccine of A13, wherein the peptide comprises the sequence KLKL5KLK (SEQ ID NO: 71) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 70).

A15. The vaccine of any one of A1-A14, further comprising one or more pharmaceutically acceptable excipient.

A16. The vaccine of any one of A1-A15, wherein the vaccine contains protamine sulphate or fragments or break-down products of PS at amounts too low to detect by HPLC, i.e., below 1 µg/mL, especially below 100 ng/mL.

A17. The vaccine of A16, wherein said protamine sulphate or fragments or break-down products of PS can be detected by mass spectroscopy or another sensitive method.

B1. A kit comprising a Zika virus vaccine of any one of A1-A15.

B2. The kit of B1, further comprising a second vaccine.

B3. The kit of B2, wherein the second vaccine is a West Nile virus vaccine, a Japanese Encephalitis virus vaccine, a Yellow Fever virus vaccine, a Dengue virus vaccine or a Chikungunya virus vaccine.

C1. A method, comprising administering a first dose of a therapeutically effective amount of the Zika virus vaccine of any one of A1-A15 to a subject in need thereof.

C2. The method of C1, further comprising administering a second dose of a therapeutically effective amount of the Zika virus vaccine.

C3. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

C4. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

C5. The method of C1 or C2, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

C6. The method of any one of C1-C5, wherein the administering results in production of Zika virus neutralizing antibodies.

D1. A method of producing a Zika virus vaccine, comprising

(i) passaging a Zika virus on Vero cells, thereby producing a culture supernatant comprising the Zika virus;

(ii) harvesting the culture medium of (i);

(iii) precipitating the harvested culture medium of (ii), thereby producing a Zika virus supernatant; and

(iv) optimally inactivating the Zika virus in the Zika virus supernatant of (iii) thereby producing an inactivated Zika virus.

D2. The method of D1, further comprising concentrating the culture medium of (ii) prior to step (iii).

D3. The method of D1 or D2, wherein the precipitating of (iii) comprises contacting the culture medium of (ii) with protamine sulfate or benzoate.

D4. The method of any one of D1-D3, further comprising (v) dialyzing the inactivated Zika virus of (iv), thereby producing a dialyzed Zika virus.

D5. The method of D4, further comprising (vi) filtering the dialyzed Zika virus of (v).

D6. The method of any one of D1-D5, wherein the inactivating is by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

D7. The method of D6, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for at least 4 days.

D8. The method of D6 or D7, wherein the chemical inactivation agent comprises formaldehyde.

D9. The method of any one of D6-D8, wherein the chemical activation is performed at about +4°C or about +22°C.

D10. The method of D8 or D9, further comprising neutralizing the formaldehyde.

D11. The method of D10, wherein the neutralizing is performed with sodium metabisulfite.

E1. The use of the optimally inactivated Zika virus vaccine of any one of A1-A15 for the treatment and/or prevention of a Zika virus infection.

E2. The use of E1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

E3. The use of E2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

E4. The use of E3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

E5. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

E6. The use of E3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

E7. The use of any one of E1-E6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

F1. A pharmaceutical composition for use in the treatment or prevention of a Zika virus infection, wherein said pharmaceutical composition comprises the optimally inactivated Zika virus vaccine of any one of A1-A15.

F2. The pharmaceutical composition of F1, wherein the inactivated Zika virus vaccine is administered in a first dose of a therapeutically effective amount to a subject in need thereof.

F3. The use of F2, wherein the inactivated Zika virus vaccine is administered in a second dose of a therapeutically effective amount to the subject.

F4. The use of F3, wherein the second dose of the inactivated Zika virus vaccine is administered about 7 days after the first dose of the Zika virus vaccine.

F5. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 14 days after the first dose of the Zika virus vaccine.

F6. The use of F3, wherein the second dose of the Zika virus vaccine is administered about 28 days after the first dose of the Zika virus vaccine.

F7. The use of any one of F1-F6, wherein the vaccine administration results in production of Zika virus neutralizing antibodies.

G1. Use of an optimized sucrose gradient centrifugation for removal of protamine sulphate from purified infectious Zika virus particles.

G2. The use according to G1, wherein said optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three further layers of sucrose solutions with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G3. A process of purification of infectious Zika virus particles, comprising the steps of:

a) providing a crude harvest (a) comprising virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

G4. The process of G3, wherein said optimized sucrose density gradient centrifugation comprises a virus comprising fraction in a 10% +/- 1% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% +/- 1% (w/w) sucrose, a second sucrose solution with 35% +/- 1% (w/w) sucrose, and a third sucrose solution with a 50% +/- 1% (w/w) sucrose.

G5. The process of any one of G3 to G4, additionally comprising a further purification step of: (d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

G6. The process of any of G3 to G5, wherein the residual host cell DNA content of the Zika virus preparation (c) is less than 10 ng/mL and the residual host cell protein content of the final virus preparation (c) is less than 100 ng/mL.

G7. The process of any of G3 to G6, wherein said crude harvest (a) comprising Zika virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

G8. The process of G7, wherein the one or more pre-purification step(s) comprises

a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or
b) digestion of host cell genomic DNA by enzymatic treatment; and/or
c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

G9. The process of any one of G3 to G8, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

G10. The process of any one of G3 to G9, wherein the enrichment of infectious Zika virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

G11. The process of any one of G7 to G10, wherein the one or more pre-purification step(s) prior to step (b) of any of G8 to G11 is performed using a filter having a pore size equal to or less than 1 µm, preferably 0.2 µm.

G12. The process of any one of G3 to G11, wherein the residual impurity of the Zika virus preparation (c) is less than 10%.

G13. The process of any one of G3 to G12, wherein the Zika virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

G14. The process of G13, wherein said cell line is a Vero cell line.

G15. The process of any one of G3 to G14, wherein said infectious Zika virus particle is an infectious virus particle that is a live virus, a live attenuated virus, a chimeric virus, a modified live virus, or a recombinant live virus.

G16. The process of any one of G3 to G15, wherein said Zika virus is preferably a strain of the Asian lineage.

G17. The process of any one of G3 to G16, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

G18. Use of the process according to any one of G3 to G17 for manufacturing a composition for immunization against a virus infection.

G19. The use according to G18, wherein said virus infection is an infection caused by a Zika virus.

Q1. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by an optimized sucrose density gradient centrifugation, wherein the optimized sucrose gradient is provided such that the protamine can be completely or almost completely separated from the virus fraction; and wherein the protamine concentration is reduced by this step to the extent that the protamine concentration in the final drug

substance is below 1 µg/ml, preferably below 0.5 µg/mL, more preferably below 0.1 µg/mL, most preferably below 0.05µg/mL.

Q2. The process of Q2, wherein the virus particles are from Zika virus.

Q3. The process of Q1 or Q2, additionally comprising the step of:
(d) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles.

Q4. The process of any of Q1 to 3, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

Q5. The process of any of Q1 to 4, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

Q6. The process of Q5, wherein the one or more pre-purification step(s) comprises
(a) filtration using a filter having a pore size equal to or less than 0.2 µm; and/or
(b) digestion of host cell genomic DNA by enzymatic treatment; and/or
(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

Q7. The process of any one of Q1 to 6, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml or 2 mg/ml.

Q8. The process of any one of Q1 to 7, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

Q9. The process of any one of Q5 to 8, wherein the one or more pre-purification step(s) prior to step (b) of any of Q5 to 8 is performed using a filter having a pore size equal to or less than 1 µm, preferably 0.2 µm.

Q10. The process of any one of Q1 to 9, wherein the residual impurity of the virus preparation (c) is less than 10%.

Q11. The process of any one of Q1 to 10, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

Q12. The process of Q11, wherein said cell line is a Vero cell line.

Q13. The process of any one of Q1 to 12, wherein the infectious virus particles is an infectious Zika virus particle that is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

Q14. The process of any one of Q1 to 13, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

Q15. The process of any one of Q1 to 14, wherein said process resulting in final virus preparation (c) or (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

Q16. Use of the process according to any one of Q1 to 15 for manufacturing a composition for immunization against a virus infection.

Q17. The use according to Q16, wherein the composition for immunization against a virus infection is an infection caused by Zika virus.

Q18. A composition comprising the virus particles obtainable or obtained by the process of any one of Q1 to 17 for treating and/or preventing an infection, such as e.g. a Zika virus infection.

Q19. A Zika virus vaccine comprising an inactivated Zika virus particle grown on vero cells, wherein the Zika virus particle is able to seroconvert a subject that is administered the Zika virus

vaccine with at least a 70% probability and comprises minor amounts of protamine sulphate, preferably below the detection limit.

Q20. The Zika virus vaccine of Q19, wherein the Zika virus particle is able to seroconvert the subject that is administered the Zika virus vaccine with at least a 80%, 85%, 90%, or 95% probability, preferably a 80% probability.

Q21. The vaccine of Q19 or 20, wherein the Zika virus particle has a RNA genome corresponding to the DNA sequence provided by any one of the nucleic acid sequences of SEQ ID NOs: 2-13, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 2-13 and able to pack a virulent Zika virus.

Q22. The vaccine of any one of Q19, 20 and 21, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 14-69 or 72, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 14-69 or 72 and able to pack a virulent Zika virus.

Q23. The vaccine of any one of Q19, 20 to 22, wherein the Zika virus obtained by culturing on Vero cells is purified by protamine sulfate precipitation and sucrose gradient centrifugation.

Q24. The vaccine of Q23, wherein the sucrose gradient centrifugation is an optimized sucrose gradient centrifugation.

Q25. The vaccine of Q24, wherein the optimized sucrose gradient centrifugation comprises a virus comprising fraction in a 10% (w/w) sucrose solution and three layers of sucrose with different densities, i.e. a first sucrose solution with 15% (w/w) sucrose solution, a second sucrose solution with 35% (w/w) sucrose solution, and a third sucrose solution with a 50% (w/w) sucrose solution.

Q26. The vaccine of any one of Q19, 20 to 25, wherein the Zika virus is inactivated by chemical inactivation, thermal inactivation, pH inactivation, or UV inactivation.

Q27. The vaccine of Q26, wherein the chemical inactivation comprises contacting the Zika virus with a chemical inactivation agent for longer than is required to completely inactivate the Zika virus as measured by plaque assay.

Q28. The vaccine of Q27, wherein the chemical inactivation comprises contacting the Zika virus with formaldehyde.

Q29. The vaccine of Q28, wherein the formaldehyde inactivation comprises contacting the Zika virus with formaldehyde for between 2-10 days.

Q30. The vaccine of any one of Q27-29, wherein the chemical activation is performed at about +4°C or about +22°C.

Q31. The vaccine of any one of Q19 to 30, further comprising an adjuvant.

Q32. The vaccine of Q31, wherein the adjuvant is an aluminum salt adjuvant.

Q33. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide or aluminium phosphate salt.

Q34. The vaccine of Q32, wherein the aluminum salt adjuvant is aluminium hydroxide with less than 1.25ppb Cu based on the final pharmaceutical composition comprising the Zika virus, preferably the inactivated Zika virus.

Q35. The vaccine of any one of Q19 to 34, further comprising one or more pharmaceutically acceptable excipient.

R1. Use of protamine, preferably a protamine salt, to separate infectious and non-infectious Zika virus particles, host cell proteins and/or undefined low molecular weight materials.

R2. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b), wherein the enrichment of infectious virus particles in the virus preparation (b) relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R3. The use of R1 or the process of R2, wherein the virus particles are from Zika virus.

R4. A process of purification of infectious Zika virus particles, comprising the steps of:

(a) providing a crude harvest (a) comprising Zika virus particles and impurities, wherein the impurities are generated from growing said virus particles on a cell substrate;

(b) reducing impurities from the crude harvest (a) by precipitation with an agent comprising protamine, preferably a protamine salt, more preferably a protamine sulphate, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);

(c) further purifying the virus preparation (b) by one or more size exclusion methods such as (i) a sucrose density gradient centrifugation, (ii) a solid-phase matrix packed in a column comprising a ligand-activated core and an inactive shell comprising pores, wherein the molecular weight cut off of the pores excludes the virus particles from entering the ligand-activated core, and wherein a molecule smaller than the molecular weight cutoff of the pores can enter the ligand-activated core and collecting the virus particles, and/or (iii) size exclusion chromatography to obtain a virus preparation (c) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (c) is less than 100 ng/mL and the residual host cell protein and the residual aggregates of infectious virus particles of the final virus preparation (c) is less than 1 μg/mL.

R5. The process of R4, wherein the residual host cell DNA of the virus preparation (c) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (c) is less than 100 ng/mL.

R6. The process of any of R2 to 5, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

R7. The process of R6, wherein the one or more pre-purification step(s) comprises

(a) filtration using a filter having a pore size equal to or less than 0.2 μm; and/or

(b) digestion of host cell genomic DNA by enzymatic treatment; and/or

(c) ultra/diafiltration using a hollow fiber membrane having a pore size equal to or greater than 300 kDa, preferably equal to or greater than 100 kDa.

R8. The process of any one of R2 to 7, wherein the concentration of protamine sulphate is 0.5 to 3 mg/ml, more preferably 1 to 2 mg/ml, more preferably 1.2 to 1.8 mg/ml, more preferably 1.4 to 1.6 mg/ml, most preferably 1.6 mg/ml.

R9. The process of any one of R2 to 8, wherein the enrichment of infectious virus particles in the virus preparation (c) or any final virus preparation relative to total virus products in the crude harvest (a) is in the range from at least 50% to 95%, preferably at least 80%.

R10. The process of any one of R6 to 9, wherein the one or more pre-purification step(s) prior to step (b) of any of R6 to 9 is performed using a filter having a pore size equal to or less than 1 µm, preferably 0.2 µm.

R11. The process of any one of R2 to 10, wherein the residual impurity of the virus preparation (c) is less than 10%.

R12. The process of any one of R2 to 11, wherein the virus is propagated in a cell line selected from the group consisting of an EB66 cell line, a Vero cell line, a Vero-αHis cell line, a HeLa cell line, a HeLa-S3 cell line, a 293 cell line, a PC12 cell line, a CHO cell line, a 3T3 cell line, a PerC6 cell line, a MDSK cell line, a chicken embryonic fibroblast cell line, a duck cell line, and a diploid avian cell line.

R13. The process of R12, wherein said cell line is a Vero cell line.

R14. The process of any one of R2 to 13, wherein the Zika virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

R15. The process of any one of R2 to 14, wherein the Zika virus is a Zika virus strain of the Asian lineage or an immunogenic variant thereof.

R16. The process of any one of R2 to 15, wherein said process resulting in final virus preparation (c) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

R17. Use of the process according to any one of R1 to 16 for manufacturing a composition for immunization against a virus infection.

R18. The use according to R17, wherein the composition for immunization against a virus infection is an infection caused by a Zika virus.

R19. A composition comprising the virus particles obtainable or obtained by the process of any one of R2 to 16 for treating and/or preventing an infection.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15 2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 02 20/2020
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EXAMINER	
CHESTNUT, BARRY A	

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
02/20/2020	ELECTRONIC

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The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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WGS_eOfficeAction@WolfGreenfield.com

PTOL-90A (Rev. 04/07)

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Application No. : 16063007
Applicant : Calzado
Filing Date : 06/15/2018
Date Mailed : 02/20/2020

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Kam Sin/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 16063007 **Drawings filed: 06/15/2018**

DRAWINGS AND BRIEF DESCRIPTION OF DRAWINGS IN NON-COMPLIANCE WITH 37 CFR 1.84(u)(1)

37 CFR 1.84(u)(1) states, "The different views must be numbered in consecutive Arabic numerals, starting with 1 … Partial views intended to form one complete view, on one or several sheets, must be identified by the same number followed by a capital letter. View numbers must be preceded by the abbreviation 'FIG.' "

Other methods of identification are improper, including Roman numerals (FIG. I, FIG, II, etc.) and alphabetic characters (FIG. A, FIG. B, etc.). Abbreviations and words other than "FIG." ("Illustration, 1" "Diagram 2," "Photo 3," etc.) are improper.

☐ Applicant must submit drawings that number the views using consecutive Arabic numerals.

☐ Applicant must amend the brief description of the drawings on Page(s) of the specification to show consecutive Arabic numerals.

NOTE: Applicant is advised that the detailed description may voluntarily be amended to identify the drawings by Arabic numerals.

☒ The drawing is continued onto a second page (or more) without proper labeling under 37 CFR 1.84(u)(1). FIG(s) 7

☐ Applicant must submit drawings on which the view numbers are preceded by the abbreviation "FIG."

☐ Applicant must amend the brief description of the drawings on Page(s) of the specification to show view numbers preceded by the abbreviation "FIG."

NOTE: Applicant is advised that the detailed description may voluntarily be amended to show view numbers preceded by the abbreviation "FIG."

Electronic Acknowledgement Receipt

EFS ID:	38816014
Application Number:	16063007
International Application Number:	
Confirmation Number:	4503
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana BARBERO CALZADO
Customer Number:	23628
Filer:	Chelsea E. Witte-Garcia/Katelyn Farmer
Filer Authorized By:	Chelsea E. Witte-Garcia
Attorney Docket Number:	I0422.70125US00
Receipt Date:	10-MAR-2020
Filing Date:	15-JUN-2018
Time Stamp:	10:05:59
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	I042270125US00-TRN-CEW.pdf	26363 3defd3741f13fde8677c0314a4fb1efba99fb0df	no	1
Warnings:		1437			

Information:					
2		I042270125US00-AMN_NCAP-CEW.pdf	30692 ef55732e38c3dbec85c828142afb9bd58e2d3c38	yes	4
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment after Notice of Allowance (Rule 312)		1	1	
	Specification		2	2	
	Drawings-other than black and white line drawings		3	3	
	Applicant Arguments/Remarks Made in an Amendment		4	4	
Warnings:					
Information:					
3	Drawings-other than black and white line drawings	I042270125US00-RDRW-CEW.pdf	1535777 91abe8551b24259cf9d1c2513f91f99cb5ecf156	no	23
Warnings:					
Information:					
4	Specification	I042270125US00-MUSPEC-CEW.pdf	514343 0d8c0934c01361454d6792d01245adfe4e44216f	no	99
Warnings:					
Information:					
5	Specification	I042270125US00-SUBSPEC-CEW.pdf	513829 7bdb747c71914edaca6e4e10efcb92bec9adcfd3	no	99
Warnings:					
Information:					
6	Miscellaneous Incoming Letter	I042270125US00-NCAP_COPY-CEW.pdf	108563 1f56584e3963ef2db1216cf71747af5ee6f160f8	no	3
Warnings:					
Information:					
		Total Files Size (in bytes):	2729567		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	16/063,007-Conf. #4503
Filing Date	June 15, 2018
First Named Inventor	Jana Barbero Calzado
Art Unit	1648
Examiner Name	B. A. Chestnut
Attorney Docket Number	I0422.70125US00

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

-Amendment in Response to Notice to File Corrected Application Papers
-Substitute Specification (clean and marked-up copies)
-Replacement Drawings (23 sheets total)
-Notice to File Corrected Application Papers (COPY)

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Chelsea E. Witte-Garcia/		
Printed name	Chelsea E. Witte-Garcia, Ph.D.		
Date	March 10, 2020	Reg. No.	75,713

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: March 10, 2020 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

REMARKS

Applicant submits herewith both marked-up and clean versions of the Substitute Specification in which the description of Figure 7 has been amended to refer to amended Figures 7A-7C, as requested in the Notice to File Corrected Application Papers. No new matter has been added.

Applicant believes no fee is due with this response. However, if a fee is due with this response, please charge our Deposit Account No. 23/2825, under Docket No. I0422.70125US00 from which the undersigned is authorized to draw.

Dated: March 10, 2020

Respectfully submitted,

By /Chelsea E. Witte-Garcia/
Chelsea E. Witte-Garcia, Ph.D.
Registration No.: 75,713
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

AMENDMENTS TO THE DRAWINGS

Applicant submits herewith Replacement Sheets (23 sheets), a full set of Figures including a clean version of amended Figure 7. Please replace the original sheets as filed with the attached Replacement Sheets. No new matter has been added.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	06/15/2018	Jana BARBERO CALZADO	I0422.70125US00	4503

23628 7590 03/19/2020

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600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER	
CHESTNUT, BARRY A	

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
03/19/2020	ELECTRONIC

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WGS_eOfficeAction@WolfGreenfield.com

Response to Rule 312 Communication	Application No. 16/063,007	Applicant(s)
	Examiner	Art Unit

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address –

1. ☒ The amendment filed on *10 March 2020* under 37 CFR 1.312 has been considered, and has been:

   a) ☒ entered.

   b) ☐ entered as directed to matters of form not affecting the scope of the invention.

   c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
   Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

   d) ☐ disapproved. See explanation below.

   e) ☐ entered in part. See explanation below.

Bernice Crittenden

Publishing Division

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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23628 7590 03/19/2020
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BOSTON, MA 02210-2206

EXAMINER	
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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR/ PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
16/063,007	06/15/2018	BARBERO CALZADO et al.	I0422.70125US00

	EXAMINER	
WOLF GREENFIELD & SACKS, P.C. 600 ATLANTIC AVENUE BOSTON, MA 02210-2206	Barry Chestnut	
	ART UNIT	PAPER
	1648	20200317

DATE MAILED: _____

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The amendment to the specification and drawing are accepted by the Examiner. Thank you.

/BARRY A CHESTNUT/ Primary Examiner, Art Unit 1648	03/17/2020

PTO-90C (Rev.04-03)

OK TO ENTER: /B.A.C/ 03/17/2020

Docket No.: I0422.70125US00
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/063,007
Confirmation No.:	4503
Filed:	June 15, 2018
For:	ZIKA VIRUS VACCINE
Examiner:	B. A. Chestnut
Art Unit:	1648

Certificate of Electronic Filing under 37 C.F.R. § 1.8
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Dated: March 10, 2020 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

AMENDMENT IN RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Notice to File Corrected Application Papers dated February 20, 2020, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Drawings begin on page 3 of this paper

Remarks begin on page 4 of this paper.

/BARRY A CHESTNUT/ 03/17/2020

OK TO ENTER: /B.A.C/ 03/17/2020

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Dated: February 13, 2020 Electronic Signature for Katelyn R. Farmer: /Katelyn R. Farmer/

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to issuance of the patent, applicant respectfully requests entry of this amendment under 37 C.F.R. § 1.312 for the above-captioned patent application.

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/063,007	05/05/2020	10639365	I0422.70125US00	4503

23628 7590 04/15/2020

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

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