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.

FEE TRANSMITTAL

Complete if known	
Application Number	16/813,862-Conf. #9700
Filing Date	March 10, 2020
First Named Inventor	Jana Barbero Calzado
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Practitioner Docket No.	I0422.70125US01

☐ 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 ($)2,080.00

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	1,720.00
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 - 20 or HP =	_____	x _____	= _____

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**
_____	_____

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
2 - 3 or HP =	_____	x _____	= _____

HP = highest number of independent claims paid for, if greater than 3.

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 ($)**	**Fee Paid ($)**
77 - 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
1051 Surcharge - Late filing fee, search fee, ...	160.00

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	July 22, 2020

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: July 22, 2020 Electronic Signature for Taryn Bollers: /Taryn Bollers/

8379314.1

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) I0422.70125US01
Application Number 16/813,862-Conf. #9700	Filed March 10, 2020
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	
[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.

[ ] attorney or agent of record. Registration number _____ .

[X] attorney or agent acting under 37 CFR 1.34. Registration number 75,713 .

/Chelsea E. Witte-Garcia/ — Signature
July 22, 2020 — Date

Chelsea E. Witte-Garcia, Ph.D. — Typed or printed name
617.646.8000 — 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: July 22, 2020 Electronic Signature for Taryn Bollers: /Taryn Bollers/

Electronic Patent Application Fee Transmittal

Application Number:	16813862
Filing Date:	10-Mar-2020
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Filer:	Chelsea E. Witte-Garcia/Taryn Bollers
Attorney Docket Number:	I0422.70125US01

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
Pages:				
Claims:				
Miscellaneous-Filing:				
LATE FILING FEE FOR OATH OR DECLARATION	1051	1	160	160
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**2080**

Electronic Acknowledgement Receipt

EFS ID:	40070140
Application Number:	16813862
International Application Number:	
Confirmation Number:	9700
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Customer Number:	23628
Filer:	Chelsea E. Witte-Garcia/Taryn Bollers
Filer Authorized By:	Chelsea E. Witte-Garcia
Attorney Docket Number:	I0422.70125US01
Receipt Date:	22-JUL-2020
Filing Date:	10-MAR-2020
Time Stamp:	10:35:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2080
RAM confirmation Number	E20207LA36485886
Deposit Account	232825
Authorized User	Taryn Bollers

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

37 CFR 1.16 (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	Applicant Response to Pre-Exam Formalities Notice	I042270125US01-NTMP-CEW.pdf	25698 415fd1596c65df2639649f37d435d2e27a154186	no	2
Warnings:					
Information:					
2		I042270125US01-PAMN-CEW.pdf	51427 ef578248153c3c6f7f57f4da577c8de493e22cee	yes	8

Multipart Description/PDF files in .zip description

Document Description	Start	End
Preliminary Amendment	1	1
Specification	2	2
Drawings-only black and white line drawings	3	3
Claims	4	7
Applicant Arguments/Remarks Made in an Amendment	8	8

Warnings:					
Information:					
3	Drawings-only black and white line drawings	I042270125US01-RDRW-CEW.pdf	1558297 1037dce2743640945a44096b793ee9f58ab496a0	no	23
Warnings:					
Information:					
4	Power of Attorney	I042270125US01-POA-CEW.pdf	1325053 f16a2b1a730dac1be75363ac3454882e42d36335	no	1
Warnings:					
Information:					

5	Miscellaneous Incoming Letter	I042270125US01-TRN-CEW.pdf	26614 e7a8760cdf356e2b84cb193930bf3c3940f36fbd	no	1
Warnings:					
Information:					
6	Specification	I042270125US01-MUSPEC-CEW.pdf	517588 91dcae4718fa0e9f3ece0303493ca53dc935f9be	no	100
Warnings:					
Information:					
7	Specification	I042270125US01-SUBSPEC-CEW.pdf	517811 69072531df80a2ad142b8faad87cb93dc98b12a6	no	100
Warnings:					
Information:					
8	Fee Worksheet (SB06)	I042270125US01-FEE-CEW.pdf	32847 7f7a793697196f4b0f781e9ad5f02f1930af24af	no	1
Warnings:					
Information:					
9	Extension of Time	I042270125US01-EXT-CEW.pdf	24469 67cb18332d5736443c600eea8fb834cc204bd4e4	no	1
Warnings:					
Information:					
10	Fee Worksheet (SB06)	fee-info.pdf	39394 0db719531ab93576dd947d19406b24d3007bb07e	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	4119198	

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.

Docket No.: I0422.70125US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/813,862
Confirmation No.:	9700
Filed:	March 10, 2020
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: July 22, 2020 Electronic Signature for Taryn Bollers: /Taryn Bollers/

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

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

Dear Sir:

In response to the Notice to File Missing Parts of Application – Filing Date Granted mailed April 22, 2020, Applicant respectfully submits the Filing Fee for the Application (as shown on accompanying Fee Transmittal), a Substitute Sequence Listing (text file), a Substitute Specification (clean and marked-up copies), Replacement Drawings (24 figures, 23 pages), a Preliminary Amendment, and a Petition for a one-month extension of time.

Please charge our Credit Card in the amount of $2,080.00 covering the fees set forth in 37 C.F.R. § 1.16(f), 1.16(a), 1.16(k), 1.16(o), and 1.17(a)(1). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed with this

Response to Notice to File Missing Parts of Application to our Deposit Account No. 23/2825, under Docket No. I0422.70125US01.

Dated: July 22, 2020

Respectfully submitted,

Electronic signature: /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

Electronic Acknowledgement Receipt

EFS ID:	40070206
Application Number:	16813862
International Application Number:	
Confirmation Number:	9700
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Customer Number:	23628
Filer:	Chelsea E. Witte-Garcia/Taryn Bollers
Filer Authorized By:	Chelsea E. Witte-Garcia
Attorney Docket Number:	I0422.70125US01
Receipt Date:	22-JUL-2020
Filing Date:	10-MAR-2020
Time Stamp:	10:38:56
Application Type:	Utility under 35 USC 111(a)

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	Sequence Listing (Text File)	I042270125US01-SEQ-CEW.txt	466222	no	-

Warnings:

Information:	
Total Files Size (in bytes):	466222

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.

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 16813862 Document Date: 07/22/2020

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 DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 16/813,862	ATTY. DOCKET NO.: I0422.70125US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: March 10, 2020	CONFIRMATION NO.: 9700
	FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet 1 of 4	GROUP ART UNIT: Not Yet Assigned	EXAMINER: Not Yet Assigned

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document Number	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
		10,639,365	B2	Barbero Calzado et al.	05-05-2020
		2013-0280295	A1	Schlegl et al.	10-24-2013
		2018-0362936	A1	Barbero Calzado et al.	12-20-2018
		2018-0362937	A1	Barbero Calzado et al.	12-20-2018
		2018-0369359	A1	Barbero Calzado et al.	12-27-2018
		2018-0371027	A1	Barbero Calzado et al.	12-27-2018
		2019-008945	A1	Barbero Calzado et al.	01-10-2019
		2020-0017555	A9	Barbero Calzado et al.	06-16-2020

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document Office/ Country	Number	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	07-13-2016	Y-Abstract
	*	WO	1999/011762		Cheil Jedang Co.	03-11-1999	
	*	WO	2001/092552	A2	Chiron Corporation	12-06-2001	
	*	WO	2013/083726		Valneva Austria GmbH	06-13-2013	
	*	WO	2016/145149	A1	The US Secretary of the Army on Behalf of the Walter Reed Army Inst of Res	09-15-2016	
	*	WO	2017/009873	A1	Bharat Biotech Int.	01-19-2017	

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.

*a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 16/063,007, filed 06-15-2018, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/813,862	ATTY. DOCKET NO.: I0422.70125US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: March 10, 2020	CONFIRMATION NO.: 9700
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	2	of	4	GROUP ART UNIT: Not Yet Assigned	EXAMINER: Not Yet Assigned

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.html.	
	*	[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]. December 24, 2009. 4 pages.	
	*	[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.	
	*	[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.	
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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.

*a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 16/063,007, filed 06-15-2018, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/813,862	ATTY. DOCKET NO.: I0422.70125US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: March 10, 2020	CONFIRMATION NO.: 9700
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	3	of	4	GROUP ART UNIT: Not Yet Assigned	EXAMINER: Not Yet Assigned

	*	DOWALL et al., A susceptible mouse model for Zika virus infection. PLOS Neglected Tropical Diseases; DOI:10.1371/journal.pntd.0004658. May 5, 2016.	
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FORM PTO-1449/A and B (modified PTO/SB/08) **INFORMATION DISCLOSURE STATEMENT BY APPLICANT**				APPLICATION NO.: 16/813,862	ATTY. DOCKET NO.: I0422.70125US01
				FILING DATE: March 10, 2020	CONFIRMATION NO.: 9700
				FIRST NAMED INVENTOR: Jana Barbero Calzado	
Sheet	4	of	4	GROUP ART UNIT: Not Yet Assigned	EXAMINER: Not Yet Assigned

	*	PINTO et al., A Temporal Role Of Type I Interferon Signaling in CD8+ T Cell Maturation during Acute West Nile Virus Infection. PLoS Pathog. 2011;7(12): e1002407. https://doi.org/10.1371/journal.ppat.1002407.	
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[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.

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Electronic Acknowledgement Receipt

EFS ID:	40075451
Application Number:	16813862
International Application Number:	
Confirmation Number:	9700
Title of Invention:	ZIKA VIRUS VACCINE
First Named Inventor/Applicant Name:	Jana Barbero Calzado
Customer Number:	23628
Filer:	Chelsea E. Witte-Garcia/Kristy Alaura
Filer Authorized By:	Chelsea E. Witte-Garcia
Attorney Docket Number:	I0422.70125US01
Receipt Date:	22-JUL-2020
Filing Date:	10-MAR-2020
Time Stamp:	15:03:41
Application Type:	Utility under 35 USC 111(a)

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	I042270125US01-TRN-CEW.pdf	26266 2d11aa34af39a678916a274b5531328849ab764c	no	1

Warnings:

Information:					
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3	Information Disclosure Statement (IDS) Form (SB08)	I042270125US01-IDS1449-CEW.pdf	49246 93f5dfabb69771b9eaa4cb53f57d5839718d9522	no	4
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Information:					
This is not an USPTO supplied IDS fillable form					
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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	16/813,862-Conf. #9700
Filing Date	March 10, 2020
First Named Inventor	Jana Barbero Calzado
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	I0422.70125US01

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):

Form PTO-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	July 22, 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: July 22, 2020 Electronic Signature for Kristy J. Alaura: /Kristy J. Alaura/

DOCKET NO.: I0422.70125US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Jana Barbero Calzado
Application No.:	16/813,862
Confirmation No.:	9700
Filed:	March 10, 2020
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: July 22, 2020 Electronic Signature for Kristy J. Alaura: /Kristy J. Alaura/

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.
*16/927,086	07-13-2020	Barbero Calzado et al.	I0422.70127US01

*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**

*a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 16/063,007, filed 06-15-2018, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

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. I0422.70125US01 from which the undersigned is authorized to draw.

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

Docket No.: I0422.70125US01
Date: July 22, 2020

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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.

Patentanmeldung Nr.	**Patent application No.**	**Demande de brevet n°**

15202585.4 / EP15202585

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP15202585.

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
p.o.

V. Joseph

Anmeldung Nr:
Application no.: 15202585.4
Demande no :

Anmeldetag:
Date of filing: 23.12.15
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Anmelder / Applicant(s) / Demandeur(s):

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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.
If no title is shown, or if the application has been filed in a non-EPO language, please refer to the description for the original title.
Si aucun titre n'est indiqué, ou si la demande a été déposée dans une langue autre qu'une langue officielle de l'OEB, se référer à la description pour le titre original.)

Alphavirus Purification

In Anspruch genommene Priorität(en) / Priority(Priorities) claimed / Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

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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

PAT015-EP-PR

ALPHAVIRUS PURIFICATION

FIELD OF THE INVENTION

The disclosure relates to methods for the purification of alphaviruses for use in vaccines.

BACKGROUND OF THE INVENTION

Regulatory agencies such as the World Health Organization establish standards and guidelines for the production of pharmaceutical compositions administered to humans, such as vaccines, that limit quantity and components of the compositions. Meeting these standards is particularly challenging with regard to production of vaccines containing biological agents, such as viruses, which must be propagated on cell substrates. Such vaccine preparations must be sterile (*i.e.*, free from independently replicating organisms) and may contain no more than 10 ng of host cell DNA per human dose, among other requirements. These standards are in place in order to ensure safety of the composition for human administration, but may introduce challenges in the development of processes used to produce such compositions.

Chikungunya virus (ChikV) is a positive-sense, single-stranded RNA virus from the genus Alphavirus, family Togaviridae. Chikungunya virus disease is mainly an outbreak disease and is associated with high attack rates. The virus is transmitted to humans via a mosquito vector and causes fever, rash, fatigue and severe polyarthralgia. Infections with ChikV generally resolve spontaneously and are not usually fatal, except in rare cases involving CNS infection, where the death rate is from 10-30%. Particularly at risk for ChikV CNS disease are infants under one year and adults over 65 years, with an infection rate of 25-fold and 6-fold higher than the general population, respectively, and with a rate of persistent disabilities estimated at between 30% and 45% (Gerardin, 2016). Furthermore, about 30 percent of all ChikV patients experience arthralgia for months to years after recovery. In some cases, neurological, renal, cardiac, respiratory or hepatic complications can also result.

There are currently no vaccines or medications available for the treatment or prevention of Chikungunya virus disease. Outbreaks in the past have occurred mainly in Africa, but the Central/East/South African (ECSA) genotype has recently expanded its geographical range, resulting in outbreaks in India, Asia, and even temperate Europe (Weaver, S., Arrival of Chikungunya Virus in the New World: Prospects for Spread and Impact on Public Health PLOS

Neglected Tropical Diseases (2014) 8(6): e2921). Although ChikV has been repeatedly imported into the Americas since 1995, no autochthonous transmission was reported until 2013 in the Caribbean. By 2015, the epidemic had spread to the mainland and caused more than 1,000,000 suspected cases in 43 countries in the Americas (Pan-American Health Organization (2015) Number of Cumulative Cases of Chikungunya Fever in the Americas). Further epidemics may been aided in part by the spread of the ChikV mosquito vector into non-endemic regions, as well as the ability of ChikV to adapt to local mosquito species (Vega-Rua et al., Chikungunya Virus Transmission Potential by Local Aedes Mosquitoes in the Americas and Europe, May 20, 2015, PLOS Neglected Tropical Diseases DOI:10.1371/journal.pntd.0003780). The high rate of contagion of Chikungunya virus disease, its potential for long-lasting complications as well as its geographical spread underscore the need for developing preventative measures, such as vaccines.

Vaccines against Chikungunya virus disease may comprise attenuated ChikV virus particles; i.e., ChikV virus particles which have been altered to reduce virulence, but maintain immunogenicity. To facilitate production, attenuated ChikV may be propagated on a cell substrate. It is known in the art that the adaptation of viruses to a particular host cell can change the surface charge of virus particles (Gardner et al., Deliberate Attenuation of Chikungunya Virus by Adaptation to Heparan Sulfate-Dependent Infectivity: A Model for Rational Arboviral Vaccine Design (2014) PLOS Neglected Tropical Diseases 8(2):e2719). As such, traditional purification technologies, such as ion exchange chromatography (see e.g. WO 0192552A2), which separate virus particles according to their surface charge may not be suited for purification of non-adapted ChikV or partial / fully adapted ChikV. Alternatively, another purification process not based on charge could be more advantageous.

SUMMARY

During the course of routine 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 provides a streamlined, gentle, reproducible and broadly-applicable process for obtaining highly-purified infectious virus particles for applications such as vaccine preparation; furthermore, the process is not dependent on the charge of the virus particle.

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

Aspects of the invention provide processes for the purification of infectious Chikungunya 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); (c) contacting the virus preparation (b) with (i) 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 cut-off of the pores can enter the ligand-activated core and collecting the virus particles to obtain a virus preparation (d), or (ii) a solid-phase matrix 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 cut-off of the pores can enter the ligand-activated core and separating the solid-phase matrix from the virus particles by filtration to produce a virus preparation (c); and further purifying the virus preparation (d) by sucrose density gradient centrifugation to obtain a virus preparation (e) 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.

In some embodiments, the residual host cell DNA of the virus preparation (e) 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 (e) is less than 10 ng/mL. In some embodiments, the residual host cell protein of the final virus

preparation (e) 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 (e) is less than 100 ng/mL.

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 Chikungunya 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 ChikV of the invention may by optionally inactivated. In some embodiments, the Chikungunya virus particle is an attenuated form of ChikV. 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 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 molecule entering the core of the solid-phase matrix has a molecular weight less than 700 kDa. In some embodiments, the ligand of the ligand-activated core of the solid-phase matrix is capable of binding the molecule that enters the ligand-activated core via cationic-, anionic-, hydrophobic- or mixed interactions. In some embodiments, the ligand of the solid-phase matrix is octylamine, diethylaminoethyl, quarternary ammonium, or sulfonate. In a preferred embodiment, the ligand of the ligand-activated core of the solid-phase matrix is octylamine. Non-limiting examples of solid-phase matrices that may be compatible with the processes described herein include, without limitation, CAPTO® Core 700, CAPTO® DEAE, CAPTO® MMC, CAPTO® Q, CAPTO® S, FRACTOGEL® TMAE, Hyx T II, Q SEPHAROSE® Fast Flow. In some embodiments, the solid-phase matrix is CAPTO® Core 700. In some embodiments, the solid-phase matrix is used as a slurry and at a final concentration between 0.5% (v/v) and 10% (v/v), preferably 0.6%, 0.7%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, most preferably 1%. In some embodiments, the solid-phase matrix is incubated with the crude harvest comprising the virus particles at refrigerated temperatures (about 2°C to 8°C) or ice bath temperatures (about 0°C) with stirring for at least 5 minutes, preferably at least 10 minutes, preferably at least 15 minutes, preferably at least 30 minutes, preferably at least 1 hour, most preferably 15 minutes.

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).

In some embodiments, the filtration step of the virus preparation (b) after contact with the solid-phase matrix 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 about 0.2 μm, such as 0.22 μm.

In some embodiments, the Chikungunya 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 Chikungunya virus infection. In a preferred embodiment, the composition is a vaccine. In preferred embodiments, the vaccine is administered to the subject once, twice or three or more times. In a preferred embodiment, the vaccine is administered once or twice. In a preferred embodiment, the vaccine is administered only once.

Other aspects provide compositions comprising the virus particles obtainable by any of the processes described herein for treating and/or preventing a Chikungunya virus infection.

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. In the drawings:

Figure 1 shows the process of flavivirus maturation in the host cell. 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 2 shows schematically the genome of the ChikV, including non-structural and structural proteins (labeled "CHIKV") as well as a schematic representation of the Δ5nsP3 attenuated Chikungunya virus used to exemplify the purification process of the current invention (labeled "Δ5nsP3"). The triangle indicates the approximate location of the deletion in the nsP3 coding region. (Figure adapted from Hallengärd et al. 2014, *supra.*)

Figure 3 shows a flow-chart of an exemplary ChikV vaccine downstream purification process from the crude harvest to formulation of the DS, a preferred embodiment of the process of the invention.

Figure 4 shows the 214 nm, 260 nm and 280 nm absorbance of the individual SGC fractions of a representative purification run of the process of the invention (A); the SEC-HPLC analysis of the final pooled fractions containing purified infectious ChikV virus particles (B); and 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 below the figure) (C). The SGC purified pool consisting of SGC fractions F7-F11 is shown in lane 12.

Figure 5 relates the SEC area (mAU*min; right axis) and $TCID_{50}$ results (log TCID50/mL; left axis) of ChikV production harvests before and after PS treatment. 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 HCP and LMW impurities (B).

Figure 6 shows electron micrographs of ChikV harvest before and after PS treatment.

Figure 7 shows 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); 10X concentrated harvest; diafiltrated harvest; PS treated material; CC700-treated material and SGC purified pool.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are processes for the purification of infectious Chikungunya virus particles, i.e., mature, functional virus particles. The processes disclosed are characterized by the removal of undesired by-products of virus production on host cells, such as non-infectious virus particles and aggregated and immature virus by-products. The processes provided herein allow the production of highly-purified live Chikungunya virus preparations comprising mostly infectious virus particles. During the course of the invention, it was observed that protamine sulphate, added to remove contaminating DNA during virus purification, resulted not only in removal of contaminating DNA, but also in the loss of a high percentage of total virus particles present in the preparation. Surprisingly, however, quantification of total infectious virus particles by TCID50 before and after PS treatment revealed that the absolute number of infectious virus particles did not change following this loss of total virus particles. This observation suggested that treatment with PS can facilitate selective removal of non-infectious, aggregated and immature viral by-products, leaving behind the infectious Chikungunya virus particles. Because by-products produced during virus growth on host cells may have different (and undesirable) immunological properties or other unwanted side-effects or safety issues, a simple and robust way to remove these by-products is of high importance for the quality and safety of the final product.

Protamines are small arginine-rich nuclear proteins, present in high amounts in the sperm of fish, which have an important role in DNA packaging during spermatogenesis. Protamine sulfate (or "protamine" or "PS") can form a stable ion pair with heparin and is thus commonly used during certain surgeries when the anti-coagulation effect of heparin is no longer needed. In large doses, protamine sulfate administered alone can also have a weak anticoagulant effect ("Protamine sulfate". Wikipedia: The Free Encyclopedia. Wikimedia Foundation, Inc. 30 Sep 2015 Web. 26 Nov 2015 <https://en.wikipedia.org/wiki/Protamine_sulfate>). Protamine Sulphate is additionally routinely used in biotechnology applications such as DNA precipitation (e.g., removal of host cell DNA from cell culture processes), purification of DNA binding proteins and retroviral-mediated gene transfer.

Protamine is obtained from salmon sperm or produced recombinantly and is used as a sulphate salt. The four major peptides, which constitute almost the entire nitrogen-containing

material in salmon protamine, have been fully characterized and found to be polypeptides of 30–32 amino acids in length, of which 21–22 residues are arginine. The average molecular mass is in the range of 4250 Da for the following sequence: PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR (SEQ ID NO: 1). Herein, protamine is also referred to as protamine salt, or preferably protamine sulphate.

The present invention relates to the use of protamine sulphate (PS) in a process of purification of a live virus, wherein the protamine sulphate facilitates the removal of impurities from a crude virus harvest, including non-infectious virus particles and aggregates. As seen in Fig. 1, virus production in the host cell can result in the release of virus products which are not mature, infectious particles, which can also be considered impurities according to the present invention. As such, the present invention also relates to the enrichment of infectious virus particles from a crude harvest containing a mix of virus particles and other viral products in various stages of maturation.

The use of protamine sulphate can follow crude cell lysis or any further step after cell lysis wherein the virus particles are further enriched or concentrated and/or other impurities are removed and/or buffer components are exchanged. The further steps may comprise filtration or concentration of the crude cell lysate.

The protamine sulphate may comprise the sequence PRRRRSSSRP VRRRRRPRVS RRRRRRGGRR RR (SEQ ID NO: 1) or a variant thereof wherein the amino acid sequence comprises from 28-35 amino acids, preferably 29-34, more preferably 30-33 amino acids, most preferably 31 or 32 amino acids. The protamine sulphate preferably comprises at least 19 arginine residues, more preferably at least 20 arginine residues, more preferably at least 21 arginine residues, even more preferably at least 22 residues, most preferably 20 or 21 arginine residues. Further, other protamine sulphate-like compounds or variants thereof may be used. Therefore, the use of the term "protamine salt" herein shall serve to encompass natural variations on SEQ ID NO: 1, preferably, but not limited to, the protamine sulphate forms.

The process comprising the use of protamine sulphate of the invention can be applied to purification of any live virus for use in pharmaceutical compositions, for example, a vaccine. The Chikungunya virus to be purified may be any live Chikungunya virus, any live attenuated Chikungunya virus or any live Chikungunya chimeric virus, preferably a live attenuated

Chikungunya virus. In one embodiment, the ChikV is also be later inactivated. In a preferred embodiment, the virus is inactivated with formaldehyde.

In a preferred embodiment, the live attenuated Chikungunya virus is the protective ChikV-ICRES1-Δ5nsP3 described by Hallengärd *et al.* (Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice (2014) J. Virology, 88(5):2858–2866). Briefly, the ChikV genome carries a positive-sense single-stranded RNA genome of 11 Kb containing two open reading frames encoding nonstructural proteins (nsP1 to nsP4) and structural proteins (C, E3, E2, 6K, and E1), respectively (see Fig. 2, top construct). The attenuated virus Δ5nsP3, based on the La Reunion ChikV strain LR2006-OPY1, was obtained by the substitution of amino acid residues 1656 to 1717 of the P1234 polyprotein with a small linker (aa sequence AYRAAAG) in the hypervariable region of the nsP3 protein (see Fig. 2, bottom construct). The Δ5nsP3 ChikV mutant was shown to be infectious, highly immunogenic and protective against challenge with wild type ChikV (Hallengärd, *et al.*, *supra* and Hallengärd, *et al.*, Prime-Boost Immunization Strategies against Chikungunya Virus (2014) J. Virology, 88(22):13333-13343). In one embodiment, the live attenuated Chikungunya virus may be a variant of the ChikV-ICRES1-Δ5nsP3 attenuated mutant virus.

A preferred embodiment of the process of the current invention is shown in Fig. 3.

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

CC700	Capto™ Core 700
ChikV	Chikungunya virus
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 Pressure Liquid Chromatography
ID	Inner diameter
LDS buffer	Lithium dodecyl sulfate sample loading buffer
MALLS	Multiangle light scattering
mAU	Milli absorbance units
PD	Process development
PFU	Plaque forming units
SEC	Size exclusion chromatography
SGC	Sucrose gradient centrifugation
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TFF	Tangential flow filtration
TCID50	Tissue culture infectious dose 50%
UF/DF	Ultrafiltration/diafiltration
rcf	Relative centrifugal force
rHSA	Recombinant human serum albumin

SEQUENCES

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

EXAMPLES

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:

Example 1: Development of a purification process for live attenuated Chikungunya virus vaccine produced in Vero cells

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 10^{12} 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
>
> Example: 4 L harvest obtained from 20 RB (850cm²) 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 cm² 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. A representative profile is shown in Fig. 4A. The ChikV Δ5nsP3 containing fractions were indicated by a peak in all three measured wavelengths (Fig. 4A, grey shaded area). 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.

As shown in Fig. 4B, there were identified two defined peaks 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 Fig. 4C. 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 Fig. 4C).

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 Fig. 5A, 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. 5B, 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 10^4 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% CO_2. 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% CO_2. 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 (Fig. 6).

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 3, 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. 5, 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 3. 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		Infectious particles
	Area [mAU*min]	Total particles/mL	% correct size (20-40 nm)	TCID50 log 10
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 Fig. 7. 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 ≤ 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 ≤ 200 ng/mL.

Endotoxin content of the SGC pool and DS was measured by Endosafe®-PTS™ 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.

What is claimed is:

CLAIMS

1. A process of purification of infectious alphavirus particles, preferably Chikungunya 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, even more preferably a recombinant protamine sulphate, to obtain a virus preparation (b);
   (c) contacting the virus preparation (b) with (i) 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 cut-off of the pores can enter the ligand-activated core and collecting the virus particles to obtain a virus preparation (d), or (ii) a solid-phase matrix 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 cut-off of the pores can enter the ligand-activated core and separating the solid-phase matrix from the virus particles by filtration to produce a virus preparation (c); and
   (d) further purifying the virus preparation (c) by sucrose density gradient centrifugation to obtain a virus preparation (d) comprising the infectious virus particles, wherein the residual host cell DNA of the virus preparation (d) is less than 100 ng/mL and the residual host cell protein of the final virus preparation (d) is less than 1 µg/mL.

2. The process of claim 1, wherein the residual host cell DNA of the virus preparation (d) is less than 10 ng/mL and the residual host cell protein of the final virus preparation (d) is less than 100 ng/mL.

3. The process of claim 1 or 2, wherein the crude harvest (a) comprising virus particles and impurities is subjected to one or more pre-purification step(s) prior to step (b).

4. The process of any one of claims 1 to 3, 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.

5. The process of any one of claims 1 to 4, wherein the concentration of protamine sulphate is 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.

6. The process of any one of claims 1 to 5, wherein the molecule entering the core of the solid-phase matrix has a molecular weight less than 700 kDa.

7. The process of any one of claims 1 to 6, wherein the ligand of the ligand-activated core of the solid-phase matrix is capable of binding the molecule that enters the ligand-activated core via cationic-, anionic-, hydrophobic- or mixed interactions.

8. The process of any one of claims 1 to 7, wherein the ligand of the ligand-activated core of the solid-phase matrix is octylamine.

9. The process of any one of claims 1 to 8, wherein the solid-phase matrix is used as a slurry and at a final concentration between 0.5% (v/v) and 10% (v/v), preferably 0.6%, 0.7%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, most preferably 1%.

10. The process of any one of claims 1 to 9, wherein the solid-phase matrix is incubated with the protamine-treated virus preparation (b) at refrigerated temperatures (2°C to 8°C) with a stirring for at least 10 minutes, preferably 15 minutes, 30 minutes or 1 hour, most preferably 15 minutes.

11. The process of any one of claims 1 to 10, wherein the enrichment of infectious virus particles in the 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%.

12. The process of any one of claims 1 to 11, wherein the filtration of step (c) of claim 1 is performed using a filter having a pore size equal to or less than 1 μm, preferably 0.2 μm.

13. The process of any one of claims 1 to 12, wherein the residual impurity of the final virus preparation is less than 10%.

14. The process of any one of claims 1 to 13, 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.

15. The process of claim 14, wherein said cell line is a Vero cell line.

16. The process of any one of claims 1 to 15, wherein the Chikungunya virus is a live virus, an attenuated live virus, a chimeric virus, a modified live virus, or a recombinant live virus.

17. The process of any one of claims 1 to 16, wherein the Chikungunya virus is the Δ5nsP3 attenuated mutant or an immunogenic variant thereof.

18. The process of any one of claims 1 to 17, wherein said process resulting in final virus preparation (d) is followed by an inactivation step, wherein the virus is inactivated preferably by formaldehyde.

19. Use of the process according to any one of claims 1 to 18 for manufacturing a composition for immunization against a Chikungunya virus infection.

20. The use according to claim 19, wherein the composition for immunization against a Chikungunya virus infection is a vaccine.

21. A composition comprising the virus particles obtainable by the process of any one of claims 1 to 18 for treating and/or preventing a Chikungunya virus infection.

ABSTRACT

Described herein are processes for purifying infectious alphavirus particles.

FIGURE 1

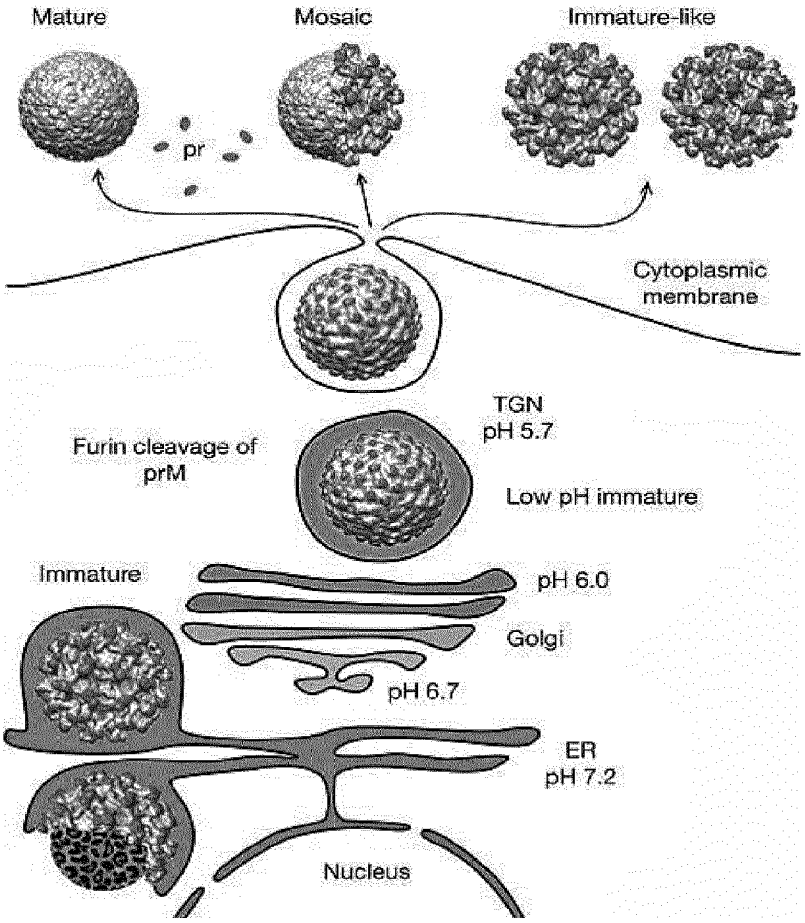

Mature
Mosaic
Immature-like
pr
Cytoplasmic membrane
TGN
pH 5.7
Furin cleavage of prM
Low pH immature
Immature
pH 6.0
Golgi
pH 6.7
ER
pH 7.2
Nucleus

FIGURE 2

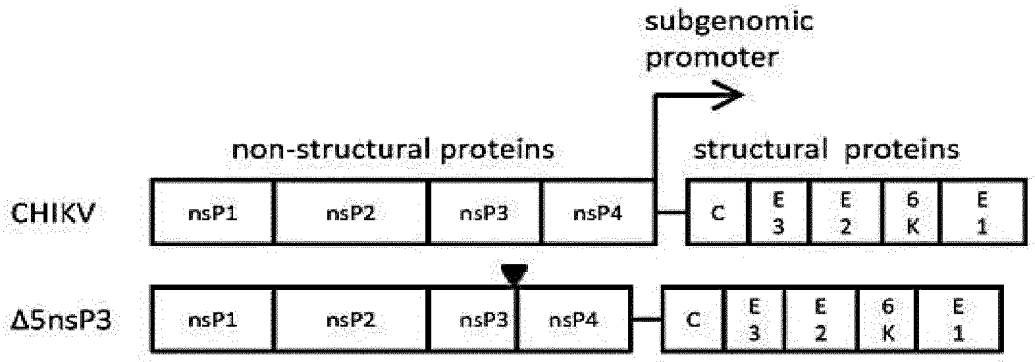

subgenomic
promoter
non-structural proteins
structural proteins
CHIKV
nsP1
nsP2
nsP3
nsP4
C
E 3
E 2
6 K
E 1
Δ5nsP3
nsP1
nsP2
nsP3
nsP4
C
E 3
E 2
6 K
E 1

FIGURE 3

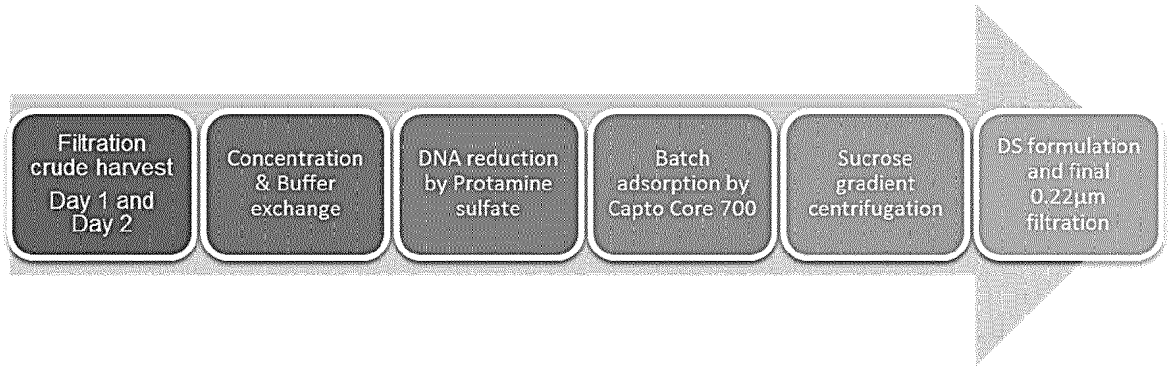

Filtration crude harvest Day 1 and Day 2
Concentration & Buffer exchange
DNA reduction by Protamine sulfate
Batch adsorption by Capto Core 700
Sucrose gradient centrifugation
DS formulation and final 0.22µm filtration

FIGURE 4A

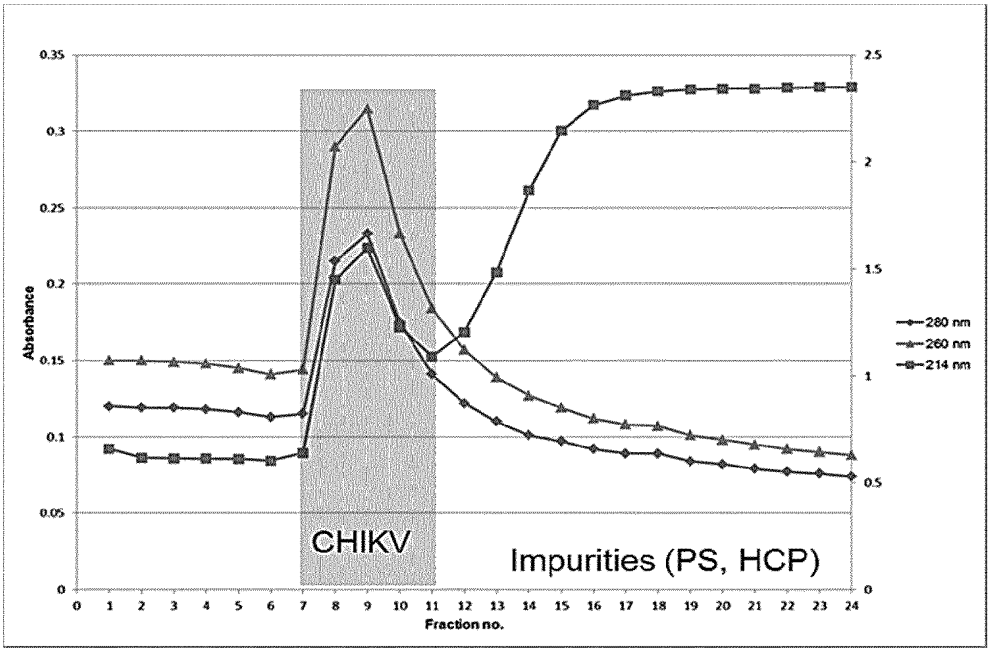


FIGURE 4B

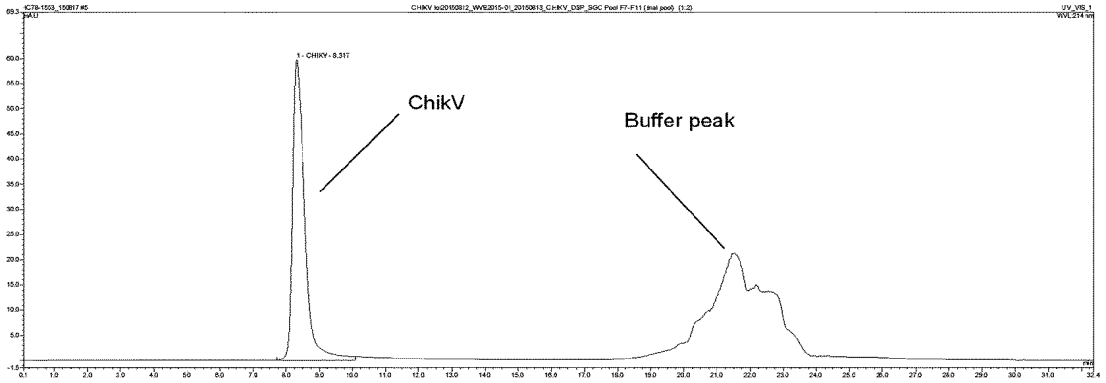
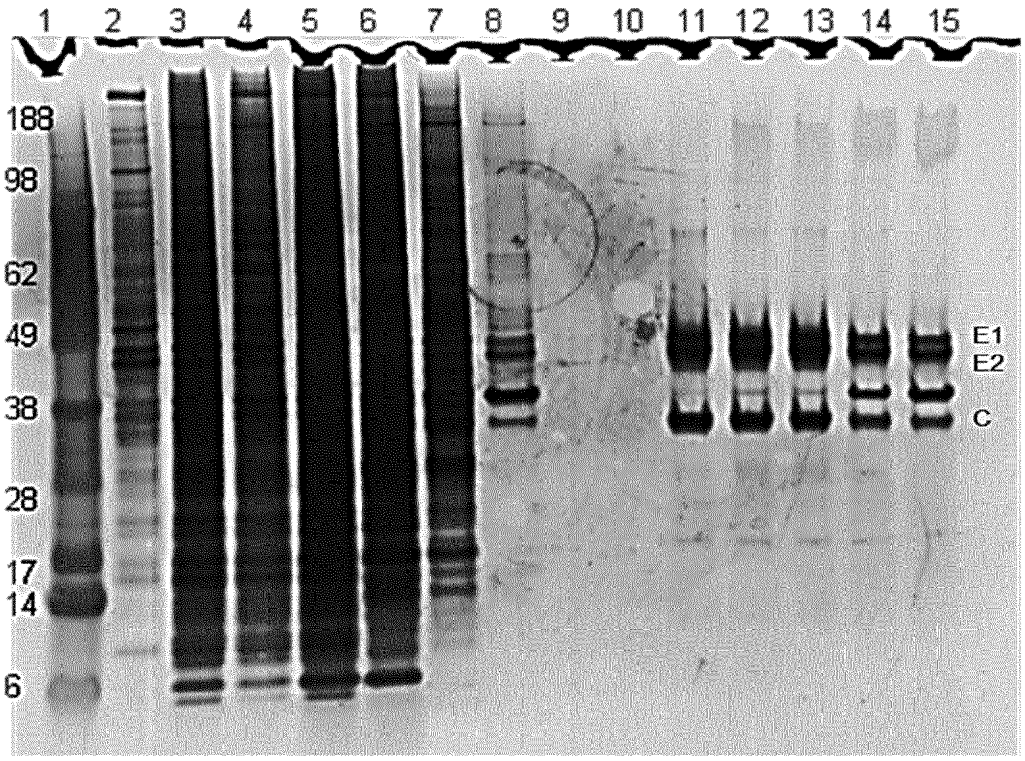


FIGURE 4C



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 5A

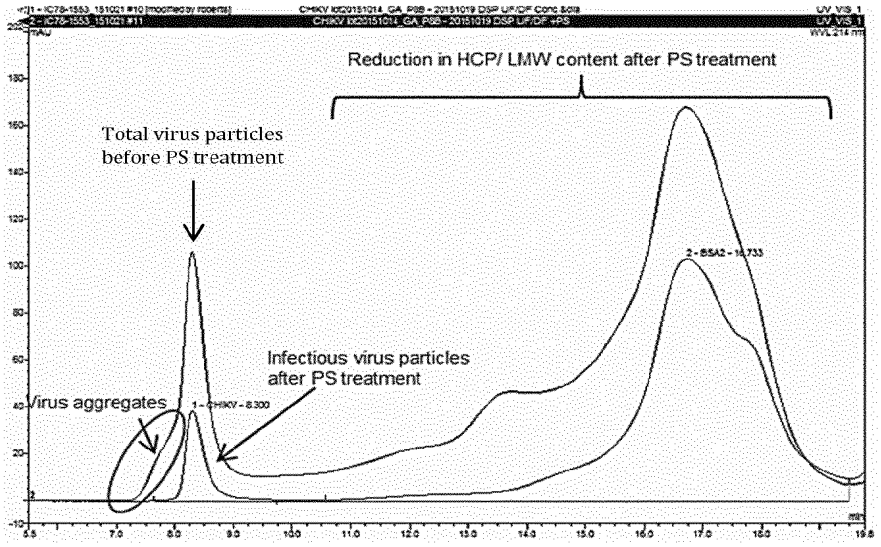


FIGURE 5B

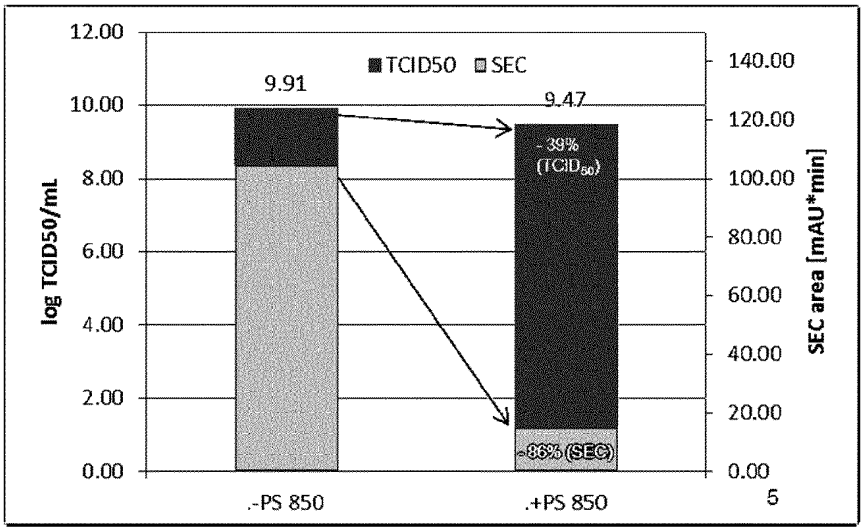


FIGURE 6

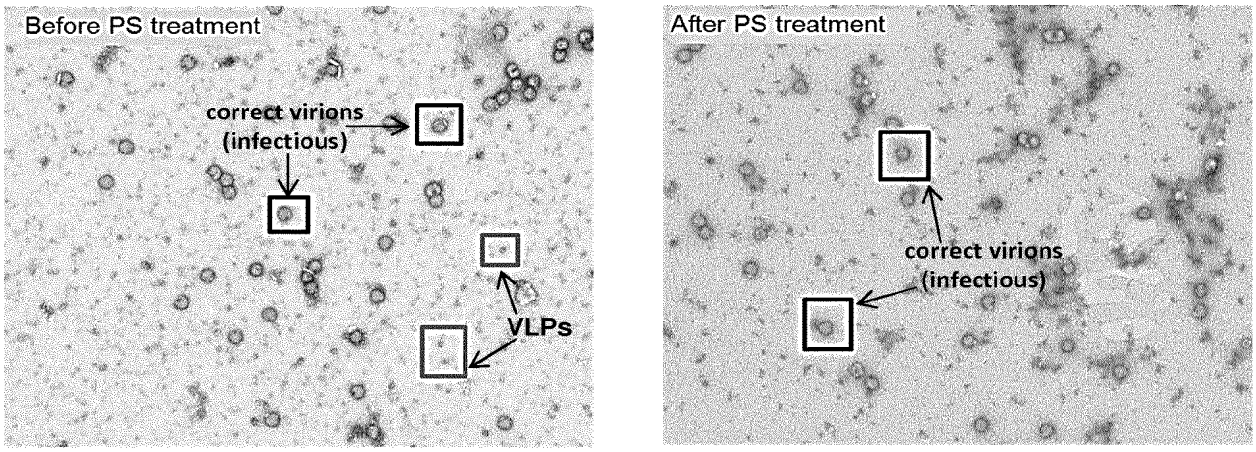
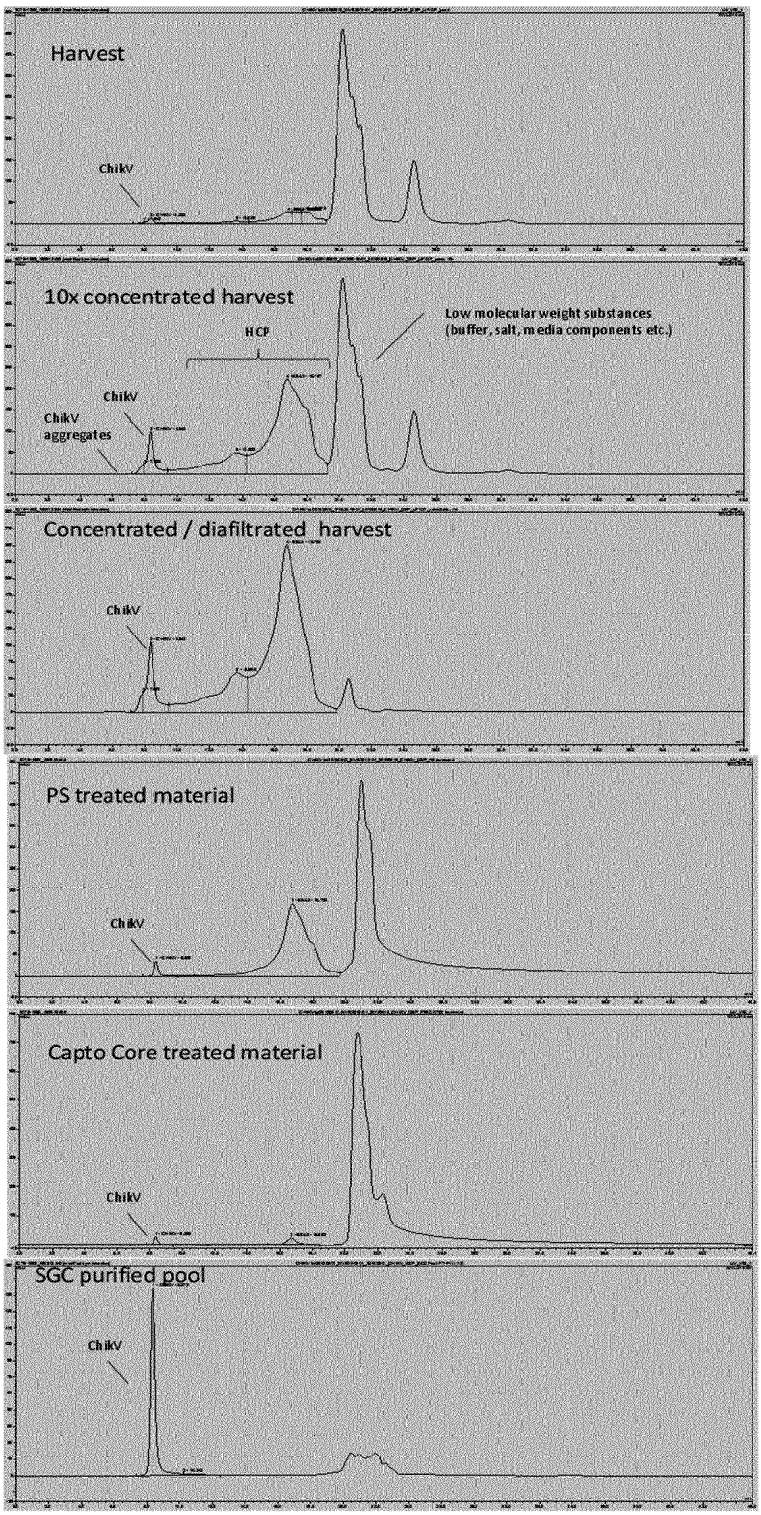

Before PS treatment
correct virions
(infectious)
VLPs
After PS treatment
correct virions
(infectious)

FIGURE 7


Harvest
ChikV
10x concentrated harvest
HCP
Low molecular weight substances
(buffer, salt, media components etc.)
ChikV
ChikV
aggregates
Concentrated / diafiltrated harvest
ChikV
PS treated material
ChikV
Capto Core treated material
ChikV
SGC purified pool
ChikV

Europäisches Patentamt
European Patent Office
Office européen des brevets

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.

Patentanmeldung Nr.	**Patent application No.**	**Demande de brevet n°**
	16161068.8 / EP16161068	

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP16161068.

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
p.o.

V. Joseph

Anmeldung Nr:
Application no.: 16161068.8
Demande no :

Anmeldetag:
Date of filing: 18.03.16
Date de dépôt :

Anmelder / Applicant(s) / Demandeur(s):

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69007 Lyon/FR

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.
If no title is shown, or if the application has been filed in a non-EPO language, please refer to the description for the original title.
Si aucun titre n'est indiqué, ou si la demande a été déposée dans une langue autre qu'une langue officielle de l'OEB, se référer à la description pour le titre original.)

INACTIVATED ZIKA VIRUS VACCINE

In Anspruch genommene Priorität(en) / Priority(Priorities) claimed / Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

Am Anmeldetag benannte Vertragstaaten / Contracting States designated at date of filing / Etats contractants désignées lors du dépôt:

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

PAT018/EP/PR

INACTIVATED ZIKA VIRUS VACCINE

FIELD OF INVENTION

The disclosure 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

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

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. In particular, the invention is directed to a Zika 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 Zika virus vaccine with at least a 70% probability, preferably an 80% probability.

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.

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 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.

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.

AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome (SEQ ID NO: 1)

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

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KU497555.1 Zika virus isolate Brazil-ZKV2015, Brazil, complete genome (SEQ ID NO: 3)

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

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

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KU527068.1 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, complete genome (SEQ ID NO: 6)

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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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TGATGAAAACCACCCATACAGGACATGGGCCTACCATGGGAGCTACGAAGCCCCCACGCAAGGATCAGCGTCTTC
CCTCGTGAACGGGGTTGTTAGACTCCTGTCAAAGCCTTGGGACGTGGTGACTGGAGTTACAGGAATAGCCATGAC

TGACACCACACCATACGGCCAACAAAGAGTCTTCAAAGAAAAAGTGGACACCAGGGTGCCAGATCCCCAAGAAGG
CACTCGCCAGGTAATGAACATAGTCTCTTCCTGGCTGTGGAAGGAGCTGGGGAAACGCAAGCGGCCACGCGTCTG
CACCAAAGAAGAGTTTATCAACAAGGTGCGCAGCAATGCAGCACTGGGAGCAATATTTGAAGAGGAAAAAGAATG
GAAGACGGCTGTGGAAGCTGTGAATGATCCAAGGTTTTGGGCCCTAGTGGATAGGGAGAGAGAACACCACCTGAG
AGGAGAGTGTCACAGCTGTGTGTACAACATGATGGGAAAAAGAGAAAAGAAGCAAGGAGAGTTCGGGAAAGCAAA
AGGTAGCCGCGCCATCTGGTACATGTGGTTGGGAGCCAGATTCTTGGAGTTTGAAGCCCTTGGATTCTTGAACGA
GGACCATTGGATGGGAAGAGAAAACTCAGGAGGTGGAGTCGAAGGGTTAGGATTGCAAAGACTTGGATACATTCT
AGAAGAAATGAATCGGGCACCAGGAGGAAAGATGTACGCAGATGACACTGCTGGCTGGGACACCCGCATTAGTAA
GTTTGATCTGGAGAATGAAGCTCTGATTACCAACCAAATGGAGGAAGGGCACAGAACTCTGGCGTTGGCCGTGAT
TAAATACACATACCAAAACAAAGTGGTGAAGGTTCTCAGACCAGCTGAAGGAGGAAAAACAGTTATGGACATCAT
TTCAAGACAAGACCAGAGAGGGAGTGGACAAGTTGTCACTTATGCTCTCAACACATTCACCAACTTGGTGGTGCA
GCTTATCCGGAACATGGAAGCTGAGGAAGTGTTAGAGATGCAAGACTTATGGTTGTTGAGGAAGCCAGAGAAAGT
GACCAGATGGTTGCAGAGCAATGGATGGGATAGACTCAAACGAATGGCGGTCAGTGGAGATGACTGCGTTGTGAA
GCCAATCGATGATAGGTTTGCACATGCCCTCAGGTTCTTGAATGACATGGGAAAAGTTAGGAAAGACACACAGGA
GTGGAAACCCTCGACTGGATGGAGCAATTGGGAAGAAGTCCCGTTCTGCTCCCACCACTTCAACAAGCTGTACCT
CAAGGATGGGAGATCCATTGTGGTCCCTTGCCGCCACCAAGATGAACTGATTGGCCGAGCTCGCGTCTCACCAGG
GGCAGGATGGAGCATCCGGGAGACTGCCTGTCTTGCAAAATCATATGCGCAGATGTGGCAGCTCCTTTATTTCCA
CAGAAGAGACCTTCGACTGATGGCTAATGCCATTTGCTCGGCTGTGCCAGTTGACTGGGTACCAACTGGGAGAAC
CACCTGGTCAATCCATGGAAAGGGAGAATGGATGACCACTGAGGACATGCTCATGGTGTGGAATAGAGTGTGGAT
TGAGGAGAACGACCATATGGAGGACAAGACTCCTGTAACAAAATGGACAGACATTCCCTATCTAGGAAAAAGGGA
GGACTTATGGTGTGGATCCCTTATAGGGCACAGACCCCGCACCACTTGGGCTGAAAACATCAAAGACACAGTCAA
CATGGTGCGCAGGATCATAGGTGATGAAGAAAAGTACATGGACTATCTATCCACCCAAGTCCGCTACTTGGGTGA
GGAAGGGTCCACACCCGGAGTGTTGTAAGCACCAATTTTAGTGTTGTCAGGCCTGCTAGTCAGCCACAGTTTGGG
GAAAGCTGTGCAGCCTGTAACCCCCCCCAGGAGAAGCTGGGAAACCAAGCTCATAGTCAGGCCGAGAACGCCATGG
CACGGAAGAAGCCATGCTGCCTGTGAGCCCCTCAGAGGACACTGAGTCAAAAAACCCCACGCGCTTGGAAGCGCA
GGATGGGAAAAGAAGGTGGCGACCTTCCCCACCCTTCAATCTGGGGCCTGAACTGGAGACTAGCTGTGAATCTCC
AGCAGAGGGACTAGTGGTTAGAGGAGACCCCCCGGAAAACGCAAAACAGCATATTGACGCTGGGAAAGACCAGAG
ACTCCATGAGTTTCCACCACGCTGGCCGCCAGGCACAGATCGCCGAACAGCGGCGGCCGGTGTGGGGAAATCCAT
GGTTTCT

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: 1-11. In some embodiments, the Zika virus has a variant genome that is at least 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: 1-11.

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

ArD157995.AHL43503.1.-/291-794 polyprotein [Zika virus]. ArD157995.AHL43503.1.-polyprotein [Zika virus]. tr|W8QFH2|W8QFH2_9FLAV Polyprotein [Zika virus] (SEQ ID NO: 12)

ISCIGVSNRDLVEGMSGGTWVDVVLEHGGCVTEMAQDKPTVDIELVTMTVSNMAEVRSYCYEASLSDMASASRCP
TQGEPSLDKQSDTQSVCKRTLGDRGWGNGCGIFGKGSLVTCSKFTCCKKMPGKSIQPENLEYRIMLPVHGSQHSG
MIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQSAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR_766.ABI54475.1.Uganda/291-788 polyprotein [Zika virus]. MR_766.ABI54475.1.Uganda polyprotein [Zika virus]. tr|C8XPB1|C8XPB1_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 13)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADT
GTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYS
LCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPP
FGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSL
FGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ARB15076.AHF49784.1.Central_African_Republic/291-788 polyprotein [Zika virus]. isol-ARB15076.AHF49784.1.Central_African_Republic polyprotein [Zika virus]. tr|W0G4H6|W0G4H6_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 14)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADT
GTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVSYS
LCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPP
FGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSL
FGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-IbH_30656.AEN75265.1.Nigeria/291-788 polyprotein, partial [Zika virus]. isol-IbH_30656.AEN75265.1.Nigeria polyprotein, partial [Zika virus]. tr|H8XX12|H8XX12_9FLAV Genome polyprotein (Fragment) [Zika virus] (SEQ ID NO: 15)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHSGADT
ETPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYS
LCTAAFTFTKVPAETLHGTVTVEVQYAGRDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPP
FGDSYIVIGVGDKKITHHWHRSGSIIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFKSL
FGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArB1362.AHL43500.1.-/291-794 polyprotein [Zika virus]. ArB1362.AHL43500.1.-polyprotein [Zika virus]. tr|W8Q6T3|W8Q6T3_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 16)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDXXXXXXNRAEVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD128000.AHL43502.1.-/291-794 polyprotein [Zika virus]. ArD128000.AHL43502.1.-polyprotein [Zika virus]. tr|W8QIT7|W8QIT7_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 17)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MXXXXXGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHRLVRKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWLKKGSSIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD158095.AHL43505.1.-/291-794 polyprotein, partial [Zika virus]. ArD158095.AHL43505.1.- polyprotein, partial [Zika virus]. tr|W8Q6T7|W8Q6T7_9FLAV Genome polyprotein (Fragment) [Zika virus] (SEQ ID NO: 18)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD158084.AHL43504.1.-/291-794 polyprotein [Zika virus]. ArD158084.AHL43504.1.- polyprotein [Zika virus]. tr|W8Q7Q3|W8Q7Q3_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 19)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ARB13565.AHF49783.1.Central_African_Republic/291-794 polyprotein [Zika virus]. isol-ARB13565.AHF49783.1.Central_African_Republic polyprotein [Zika virus]. tr|W0G1G4|W0G1G4_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 20)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ARB7701.AHF49785.1.Central_African_Republic/291-794 polyprotein [Zika virus]. isol-ARB7701.AHF49785.1.Central_African_Republic polyprotein [Zika virus]. tr|W0G5P7|W0G5P7_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 21)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGVHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-ArD_41519.AEN75266.1.Senegal/291-794 polyprotein, partial [Zika virus]. isol-ArD_41519.AEN75266.1.Senegal polyprotein, partial [Zika virus]. tr|H8XX13|H8XX13_9FLAV Genome polyprotein (Fragment) [Zika virus] (SEQ ID NO: 22)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR766-NIID.BAP47441.1.Uganda/291-794 polyprotein [Zika virus]. MR766-NIID.BAP47441.1.Uganda polyprotein [Zika virus]. tr|A0A090BCI3|A0A090BCI3_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 23)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MTVNDIGYETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

LC002520.1/326-829 Zika virus genomic RNA, strain: MR766-NIID, Uganda, polyprotein, from complete genome (SEQ ID NO: 24)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MTVNDIGYETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

isol-MR_766.AEN75263.1.Uganda/291-794 polyprotein, partial [Zika virus]. MR_766.AMK02027.1.Uganda polyprotein [Zika virus]. isol-MR_766.AEN75263.1.Uganda polyprotein, partial [Zika virus]. MR_766.AMK02027.1.Uganda polyprotein [Zika virus]. tr|H8XX10|H8XX10_9FLAV (SEQ ID NO: 25)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGYETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGKLFSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

ArD7117.AHL43501.1.-/291-794 polyprotein [Zika virus]. ArD7117.AHL43501.1.-polyprotein [Zika virus]. tr|W8R1T1|W8R1T1_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 26)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG

MIVNDIGHETDENRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRL
KGVSYSLCTAVCTAAKVPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

AY632535.2/326-825 NC_012532.1 Zika virus strain MR 766, Uganda, polyprotein, from complete genome (SEQ ID NO: 27)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIGYETDEDRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGA
DTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVS
YSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELD
PPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFK
SLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

MR_766.AAV34151.1.Uganda/291-790 polyprotein [Zika virus] MR_766.AAV34151.1.Uganda polyprotein [Zika virus]. MR_766.YP_002790881.1.Uganda flavivirus polyprotein [Zika virus]. MR_766.YP_002790881.1.Uganda flavivirus polyprotein [Zika virus]. tr|Q32ZE1|Q32ZE1_9FL (SEQ ID NO: 28)

IRCIGVSNRDFVEGMSGGIWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIGYETDEDRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGA
DTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVS
YSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELD
PPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFK
SLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

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

IRCIGVSNRDFVEGMSGGIWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFTCSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIGYETDEDRAKVEVTPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGA
DTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLFSGHLKCRLKMDKLRLKGVS
YSLCTAAFTFTKVPAETLHGTVTVEVQYAGTDGPCKIPVQMAVDMQTLTPVGRLITANPVITESTENSKMMLELD
PPFGDSYIVIGVGDKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVFNSLGKGIHQIFGAAFK
SLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVMIFLSTAVSA

KU681081.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/THA/2014/SV0127- 14, Thailand, polyprotein, from complete genome (SEQ ID NO: 30)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HTGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand/291-794 polyprotein [Zika virus]. isol-Zika_virus%H.sapiens-tc%THA%2014%SV0127-_14.AMD61710.1.Thailand polyprotein [Zika virus] (SEQ ID NO: 31)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HTGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITEGTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGVLNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

CK-ISL_2014.AIC06934.1.Cook_Islands/1-504 E protein, partial [Zika virus]. tr|A0A060H177|A0A060H177_9FLAV E protein (Fragment) OS=Zika virus GN=E PE=4 SV=1 (SEQ ID NO: 32)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal/291-794 polyprotein [Zika virus]. Natal_RGN.AMB18850.1.Brazil:_Rio_Grande_do_Norte,_Natal polyprotein [Zika virus] (SEQ ID NO: 33)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Si323.AMC37200.1.Colombia/1-504 polyprotein, partial [Zika virus]. isol-Si322.AMC37201.1.Colombia polyprotein, partial [Zika virus]. isol-Si322.AMC37201.1.Colombia polyprotein, partial [Zika virus]. isol-Si323.AMC37200.1.Colombia polyprotein, partial [Zika virus] (SEQ ID NO: 34)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU707826.1/317-820 Zika virus isolate SSABR1, Brazil, complete genome (SEQ ID NO: 35)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU509998.1/326-829 Zika virus strain Haiti/1225/2014, Haiti, polyprotein, from complete genome (SEQ ID NO: 36)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-GDZ16001.AML82110.1.China/291-794 polyprotein [Zika virus]. isol-GDZ16001.AML82110.1.China polyprotein [Zika virus] (SEQ ID NO: 37)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

BeH819015.AMA12085.1.Brazil/291-794 polyprotein [Zika virus]. BeH819966.AMA12086.1.Brazil polyprotein [Zika virus]. isol-SSABR1.AMH87239.1.Brazil polyprotein [Zika virus]. isol-SSABR1.AMH87239.1.Brazil polyprotein [Zika virus]. BeH819966.AMA12086.1.Brazil polyprotein [Zika virus] (SEQ ID NO: 38)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique/291-794 polyprotein [Zika virus]. MRS_OPY_Martinique_PaRi_2015.AMC33116.1.Martinique polyprotein [Zika virus] (SEQ ID NO: 39)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU501215.1/308-811 Zika virus strain PRVABC59, Puerto Rico, polyprotein, from complete genome (SEQ ID NO: 40)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW

HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

Haiti%1225%2014.AMB37295.1.Haiti/291-794 polyprotein [Zika virus]. Haiti%1225%2014.AMB37295.1.Haiti polyprotein [Zika virus] (SEQ ID NO: 41)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU527068.1/308-811 Zika virus strain Natal RGN, Brazil: Rio Grande do Norte, Natal, polyprotein, from complete genome (SEQ ID NO: 42)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106027.ALX35662.1.Suriname/5-508 polyprotein, partial [Zika virus]. isol-Z1106027.ALX35662.1.Suriname polyprotein, partial [Zika virus] (SEQ ID NO: 43)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-FLR.AMM39804.1.Colombia:_Barranquilla/291-794 polyprotein [Zika virus]. isol-FLR.AMM39804.1.Colombia:_Barranquilla polyprotein [Zika virus] (SEQ ID NO: 44)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada/254-757 polyprotein, partial [Zika virus]. PLCal_ZV_isol-From_Vero_E6_cells.AHL37808.1.Canada polyprotein, partial [Zika virus]. tr|A0A096XFQ2|A0A096XFQ2_9FLAV Genome polyprotein (Fragment) [Zika virus] (SEQ ID NO: 45)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

BeH818995.AMA12084.1.Brazil/291-794 polyprotein [Zika virus].
BeH818995.AMA12084.1.Brazil polyprotein [Zika virus] (SEQ ID NO: 46)
BeH815744.AMA12087.1.Brazil polyprotein [Zika virus]. BeH815744.AMA12087.1.Brazil polyprotein [Zika virus].

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

H%PF%2013.AHZ13508.1.French_Polynesia/291-794 polyprotein [Zika virus].
H%PF%2013.AHZ13508.1.French_Polynesia polyprotein [Zika virus].
tr|A0A024B7W1|A0A024B7W1_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 47)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

PRVABC59.AMC13911.1.Puerto_Rico/291-794 polyprotein [Zika virus].
PRVABC59.AMC13911.1.Puerto_Rico polyprotein [Zika virus] (SEQ ID NO: 48)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU321639.1/326-829 Zika virus strain ZikaSPH2015, Brazil, polyprotein, from complete genome (SEQ ID NO: 49)

IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

ZikaSPH2015.ALU33341.1.Brazil/291-794 polyprotein [Zika virus]. ZikaSPH2015.ALU33341.1.Brazil polyprotein [Zika virus] (SEQ ID NO: 50)

IRCIGVSNRDFVEGMSGGTWVDIVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

103344.AMC13912.1.Guatemala/291-794 polyprotein [Zika virus]. 103344.AMC13912.1.Guatemala polyprotein [Zika virus]. 8375.AMC13913.1.Guatemala polyprotein [Zika virus]. 8375. AMC13913.1.Guatemala polyprotein [Zika virus]. (SEQ ID NO: 51)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEIRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Brazil-ZKV2015.AMD16557.1.Brazil/291-794 polyprotein [Zika virus]. isol-Brazil-ZKV2015.AMD16557.1.Brazil polyprotein [Zika virus] (SEQ ID NO: 52)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

KU497555.1/308-811 Zika virus isolate Brazil-ZKV2015, Brazil, polyprotein, from complete genome (SEQ ID NO: 53)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGTQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-ZJ03.AMM39806.1.China/291-794 polyprotein [Zika virus]. isol-ZJ03.AMM39806.1.China polyprotein [Zika virus] (SEQ ID NO: 54)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM

LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGARRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-FSS13025.AFD30972.1.Cambodia/291-794 polyprotein, partial [Zika virus]. isol-FSS13025.AFD30972.1.Cambodia polyprotein, partial [Zika virus]. tr|H9A910|H9A910_9FLAV Genome polyprotein (Fragment) [Zika virus] (SEQ ID NO: 55)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106032.ALX35660.1.Suriname/291-794 polyprotein, partial [Zika virus]. isol-Z1106032.ALX35660.1.Suriname polyprotein, partial [Zika virus] (SEQ ID NO: 56)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106033.ALX35659.1.Suriname/291-794 polyprotein [Zika virus]. isol-Z1106033.ALX35659.1.Suriname polyprotein [Zika virus] (SEQ ID NO: 57)

IRCIGVSNRDFVEGMSGGIWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNAKNGSISLMCLALGGVLIFLSTAVSA

isol-BeH828305.AMK49165.1.Brazil/291-794 polyprotein [Zika virus]. isol-BcH828305.AMK49165.1.Brazil polyprotein [Zika virus] (SEQ ID NO: 58)

IRCIGVSNRDFVEGMSGGIWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDTQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-GD01.AMK79468.1.China/291-794 polyprotein [Zika virus]. isol-GD01.AMK79468.1.China polyprotein [Zika virus] (SEQ ID NO: 59)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNGTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL

KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-Z1106031.ALX35661.1.Suriname/291-794 polyprotein, partial [Zika virus]. isol-Z1106031.ALX35661.1.Suriname polyprotein, partial [Zika virus] (SEQ ID NO: 60)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVLAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

ACD75819.1.Micronesia/291-794 polyprotein [Zika virus]. ACD75819.1.Micronesia polyprotein [Zika virus]. tr|B3U3M3|B3U3M3_9FLAV Genome polyprotein [Zika virus] (SEQ ID NO: 61)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPAVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

KU681082.3/308-811 Zika virus isolate Zika virus/H.sapiens-tc/PHL/2012/CPC-0740, Philippines, polyprotein, from complete genome (SEQ ID NO: 62)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines/291-794 polyprotein [Zika virus]. isol-Zika_virus%H.sapiens-tc%PHL%2012%CPC-0740.AMD61711.1.Philippines polyprotein [Zika virus] (SEQ ID NO: 63)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

isol-BeH823339.AMK49164.2.Brazil/291-794 polyprotein [Zika virus]. isol-BeH823339.AMK49164.2.Brazil polyprotein [Zika virus] (SEQ ID NO: 64)
IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVSTTVSNMAEVRSYCYEATISDIASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG

MIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTAVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

isol-P6-740.AEN75264.1.Malaysia/291-794 polyprotein, partial [Zika virus]. isol-P6-740.AEN75264.1.Malaysia polyprotein, partial [Zika virus]. tr|H8XX11|H8XX11_9FLAV Genome polyprotein (Fragment) [Zika virus] (SEQ ID NO: 65)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MIVNDXGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPW
HAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGDKKITHHWXRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFG
AAFKSLFGGMSWFSQILIGTLLVWLGLNTKNGSISLTCLALGGVLIFLSTAVSA

KU744693.1/326-829 Zika virus isolate VE_Ganxian, China, polyprotein, from complete genome (SEQ ID NO: 66)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MLVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPW
HAGAATGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETVDGTVTVEGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSG

isol-VE_Ganxian.AMK79469.1.China/291-794 polyprotein [Zika virus]. isol-VE_Ganxian.AMK79469.1.China polyprotein [Zika virus] (SEQ ID NO: 67)

IRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTAMAQDKPTVDIELVTTTVSNMAEVRSYCYEASISDMASDSRCP
TQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMTGKSIQPENLEYRIMLSVHGSQHSG
MLVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHWLAHKEWFHDIPLPW
HAGAATGTPHWNNKEALVEFKDAHAKRQTVVVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRL
KGVSYSLCTAAFTFTKIPAETVDGTVTVEGQYGGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMM
LELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIIG
AAFKSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSG

In some embodiments, the Zika virus has an envelope (E) protein with an amino acid sequence provided by any one of SEQ ID NO: 12-67. In some embodiments, the E protein sequence is at least 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to the E protein sequence provided by any one of the amino acid sequences SEQ ID NO: 12-67.

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.

Aspects of the disclosure relate to methods of producing a Zika 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 Zika virus, the culture medium is inoculated with Zika virus and the cells are incubated with the Zika 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 Zika virus in Vero cells. As used herein, the terms "passage" or "passaging" refer to infecting a population of Vero cells with Zika virus and subsequently inoculating a second population of Vero cells with Zika 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 Zika 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, Zika virus obtained from a single plaque is used to inoculate another population of cells.

In some embodiments, the Zika 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 Zika virus is passaged in Vero cells at least 4 times. In some embodiments, the Zika virus is passaged in Vero cells at least 30 times.

In some embodiments, the Vero cells are incubated for at least 2 days after inoculation with the Zika virus 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 20 days after inoculation with the Zika 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 Zika virus in Vero cells may result in generation of a Vero cell adapted Zika virus strain.

The culture medium from the infected Vero cells may be harvested (collected) to obtain the Zika 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 8 or 9 days post infection, for example, and result in a high yield of virus per unit cell culture. The harvested culture medium can be stored at +4°C prior to purification of the virus from the culture medium.

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 Zika 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 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. In some embodiments, the precipitated culture medium is centrifuged to collect precipitated material and the supernatant containing the Zika virus, referred to as a "Zika virus supernatant," is collected.

The Zika virus supernatant may be further purified after precipitation, for example density gradient ultracentrifugation. In some embodiments, the Zika virus supernatant is

further purified by sucrose gradient. Fractions may be collected from the sucrose gradients and assayed for presence of the Zika 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 Zika virus may be pooled based on titer of the virus and level of other impurities. The level or amount of impurities present in the Zika virus supernatant can be estimated by testing for Vero cell DNA and/or Vero cell protein.

To achieve a safe vaccine or composition for the administration to subjects, the Zika virus supernatant is inactivated. 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 Zika 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 Zika 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 Zika virus that is not subjected to inactivation.

A variety of methods are known in the art for inactivating viruses. In some embodiments, the Zika 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 Zika 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 Zika virus is inactivated for a period of time that is longer than is required to completely inactivate the Zika virus. In some embodiments, the Zika virus supernatant is inactivated for the number of days required to inactivate the virus plus at least one additional day. Samples of the Zika 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 Zika

virus can be determined, and a longer period of time is selected to ensure complete inactivation.

In some embodiments, the Zika 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 Zika 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 Zika 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 Zika 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.

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 Zika virus is inactivated by thermal inactivation. In some embodiments, the thermal inactivation involves exposing the Zika virus to heat, such as dry heat or vapor heat, for a period of time. In some embodiments, the thermal inactivation involves exposing the Zika 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 Zika 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 Zika virus to temperatures of about +56°C for 60 minutes.

In some embodiments, the Zika 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 Zika 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 Zika virus is inactivated using UV inactivation. UV inactivation involves exposing the Zika 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 Zika 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 Zika virus is dialyzed with PBS. In addition or alternatively, the inactivated Zika 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," and "preventing," include the administration of a Zika 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.

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.

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: 68). 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: 68) and the nucleic acid sequence $(dIdC)_{13}$ (SEQ ID NO: 69), 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.

EXAMPLES

Example 1: Adaptation of Zika Virus in Vero cells

Vero cell monolayers are inoculated with Zika virus. The infected cell cultures are grown in 25 cm^2 culture flasks containing 5 mL of nutrient media consisting of Eagle's minimal essential media (EMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of about 5% CO_2 in air and at a temperature no higher than about +35°C. Viral replication is monitored by microscopic observation of cytopathic effect (CPE) and various assays for the presence of viral antigen including hemadsorption assay (HA), plaque assay, and enzyme linked immunoadsorbant assay (ELISA). Zika virus is harvested at day *e.g.* day 1 and/or 2 or other day, e.g. 3, 4, 5, 6, 7, 8, 9, 10 post infection when the culture showed peak of virus titer, and culture media is clarified by centrifugation to remove cell debris. The clarified supernatant is used to inoculate additional Vero cell monolayers in order to amplify the virus. Subsequent serial passages are conducted up to 30 passages by successive virus infection, titration, and plaque-purification as described above. The viral yield in the culture media is quantified by plaque assay.

Example 2: Virus growth and purification

For viral production and subsequent purification, Vero cells are grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS). Roller bottle cultures of Vero cell monolayers are infected with Zika virus at a multiplicity of infection (moi) of 0.01 to 1 plaque forming units (pfu) per cell. After 2 hours of virus adsorption, the cultures are washed 3 times with PBS and fed with EMEM without FBS and incubated at +35°C with 5% CO_2. Infected Vero cell cultures are incubated until the virus titer reaches a desired level.

The culture medium is harvested and may be pooled from independent harvest days and then centrifuged at 8,000 rpm for 15 minutes in standard centrifuge. The supernatants are then filtered through a 0.45 μm filter. Virus culture supernatants are concentrated by ultrafiltration (Ultrasette, Filtron, 100k) or precipitation with PEG. The virus precipitated by PEG is collected by centrifugation and resuspended in PBS or STE (10 mM Tris pH 7.2, 1 mM EDTA, 150 mM NaCl) buffer. Retentate after ultrafiltration is concentrated to 250 mL and the cassette is washed with 100 mL of PBS. Virus concentrates are precipitated by

chilling in the ice for 2 hours after adding 0.5-2 mg/mL of protamine sulfate. The precipitated concentrates are centrifuged at 10,000 rpm for 5 minutes in standard centrifuge to obtain the supernatant containing the virus. The concentrated viruses are purified by ultra-centrifugation on sucrose gradients at 38,000 *g* for 18 hours.

Fractions are subjected to analysis by SDS-PAGE. Envelope antigens (E) may be detected by Western blotting with anti-Zika virus monoclonal antibody. Virus positive fractions are pooled and assayed for protein concentration, for example by Lowry method. Purified virus is diluted with two volumes of PBS and then filtered through a 0.22 μm filter.

The virus preparations are compared for relative purity using specific activity measurements (*i.e.*, pfu/mg protein). The purity of the Zika virus preparations are estimated by assessing the preparations for any Vero cell DNA or protein.

Example 3: Virus Inactivation

Purified Zika virus preparations are inactivated with formaldehyde for preparation of inactivated vaccine. Inactivation with 0.018% formaldehyde may be carried out at +22°C or +4°C for at least 2 days, samples from the viral inactivation may be taken at different time intervals to assess for viral viability by plaque assay. The antigenicity of the virus can also be monitored during inactivation by testing samples with antigen dot blot assay and probing with a polyclonal antiserum.

After compete viral inactivation, the formaldehyde is neutralized by the addition of 0.038% of sodium metabisulfite. Dialysis is performed using PBS and the preparations are then filtered through a 0.22 μm filter.

Example 4: Immunogenicity of Vero adapted Zika virus

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.

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: 1-11, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 1-11 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: 12-67, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 12-67 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: 68) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 69).

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 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.

2. The Zika virus vaccine of claim 1, 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.

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: 1-11, or a variant nucleic acid sequence that is at least 88% identical to any one of SEQ ID NOs: 1-11 and able to pack a virulent Zika virus.

4. The vaccine of any one of claims 1-3, wherein the Zika virus particle has an E protein selected from the amino acid sequences provided by any one of SEQ ID NOs: 12-67, or a variant amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 12-67 and able to pack a virulent Zika virus.

5. The vaccine of any one of claims 1-4, 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 5-8, wherein the chemical activation is performed at about +4°C or about +22°C.

10. The vaccine of any one of claims 1-9, 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 10-12, 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: 68) and the I-ODN comprises oligo-d(IC)13 (SEQ ID NO: 69).

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

ABSTRACT

Described herein are Zika virus vaccines and compositions and methods of producing and administering said vaccines to subjects in need thereof.

5

Figure 1

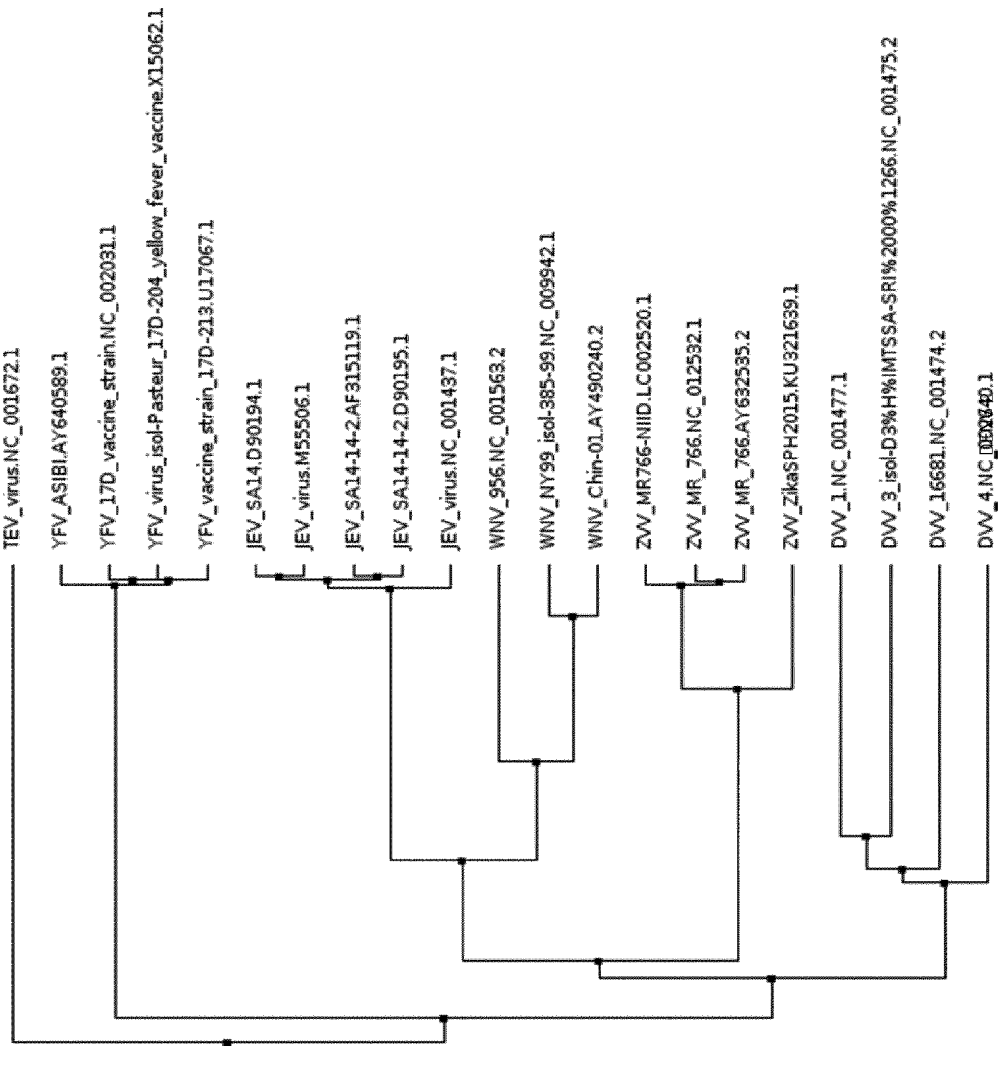

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JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
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WNV_Chin-01.AY490240.2
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
ZVV_MR_766.AY632535.2
ZVV_ZikaSPH2015.KU321639.1
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Figure 2

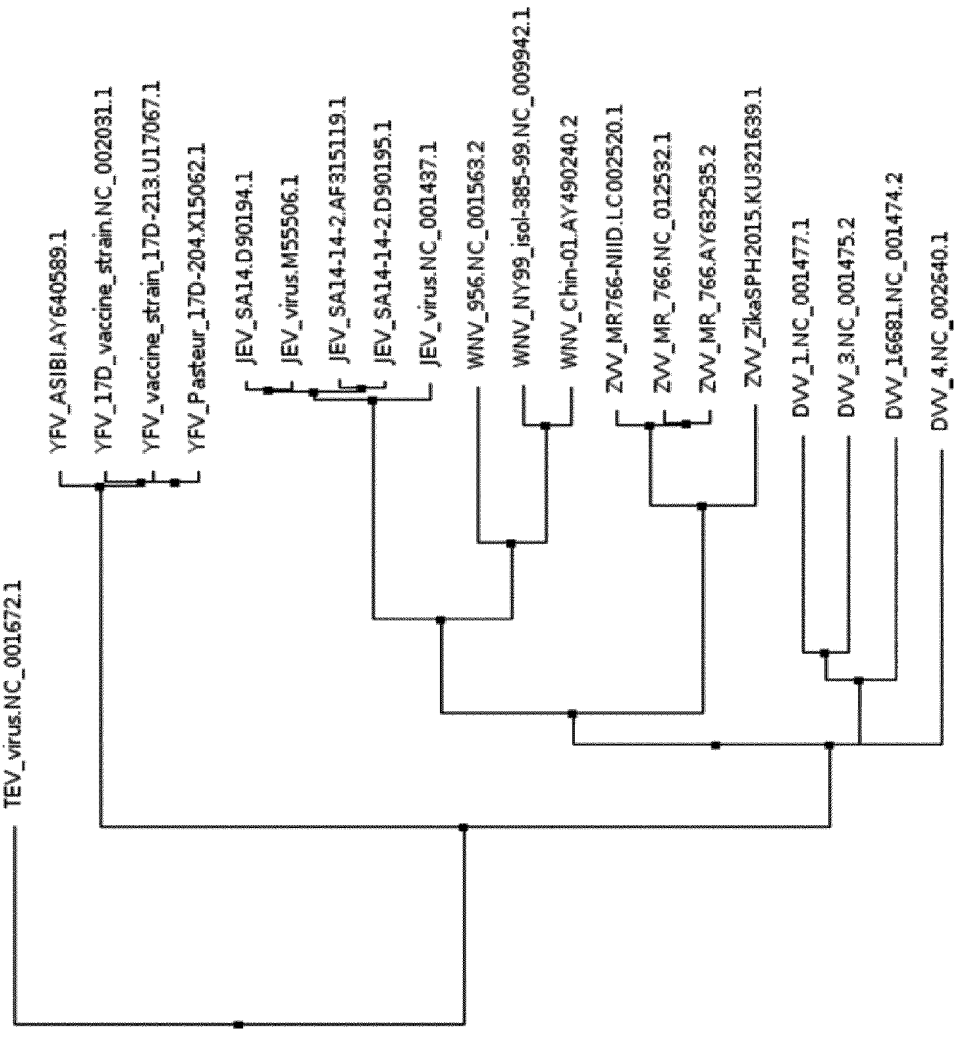

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JEV_SA14.D90194.1
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DW_3.NC_001475.2
DW_16681.NC_001474.2
DW_4.NC_002640.1

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

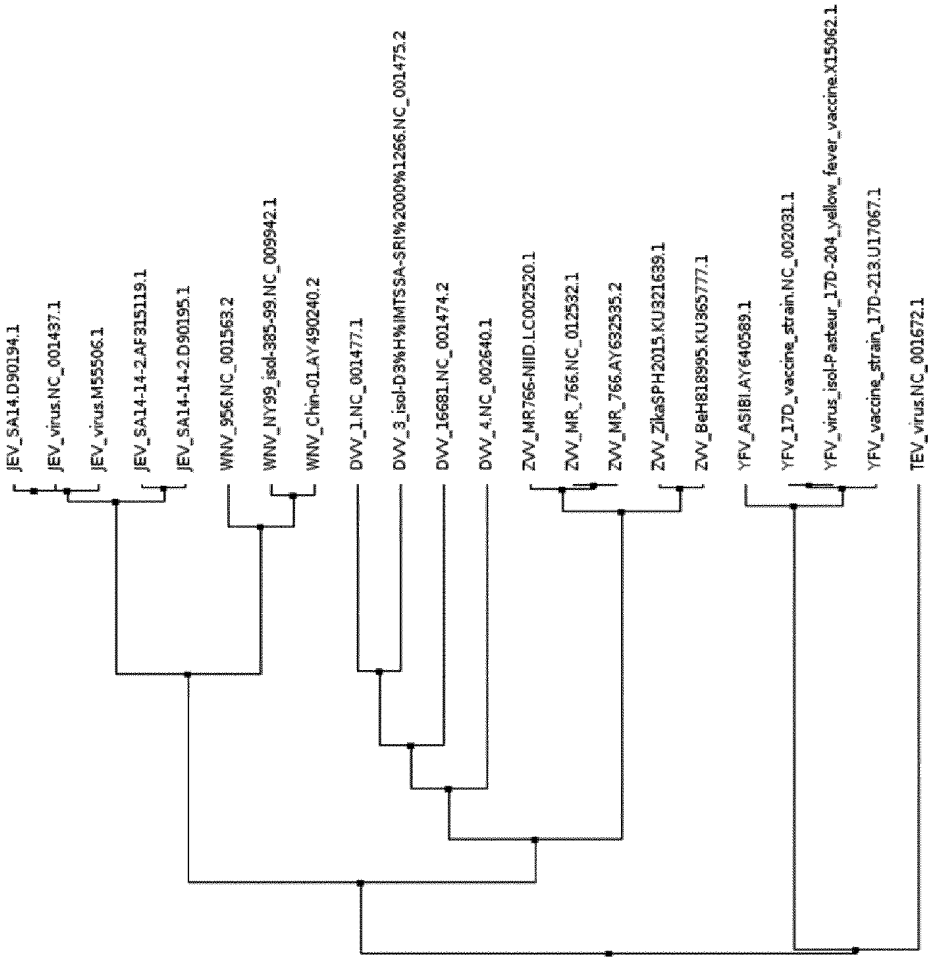

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TEV_virus.NC_001672.1

Figure 5

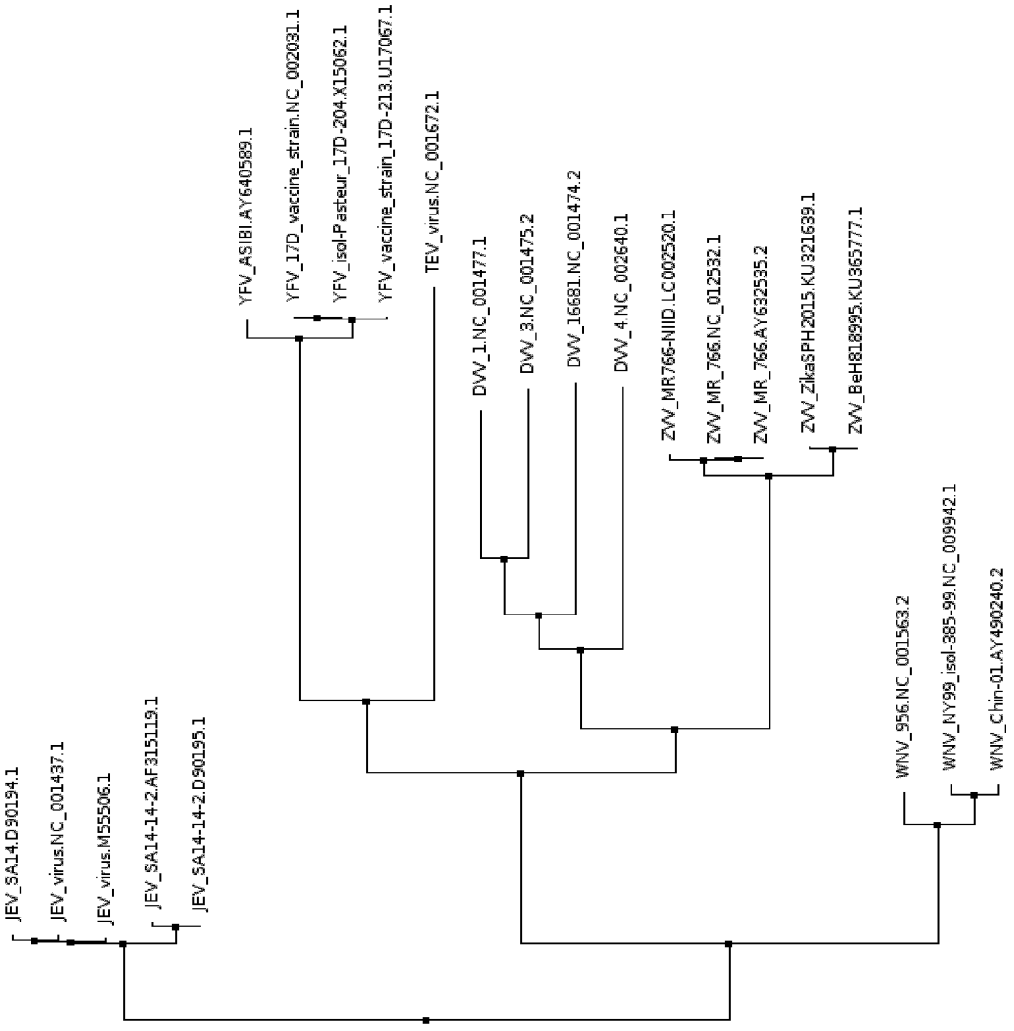

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YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1
DVV_1.NC_001477.1
DVV_3.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
ZVV_MR_766.NC_012532.1
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WNV_Chin-01.AY490240.2

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)

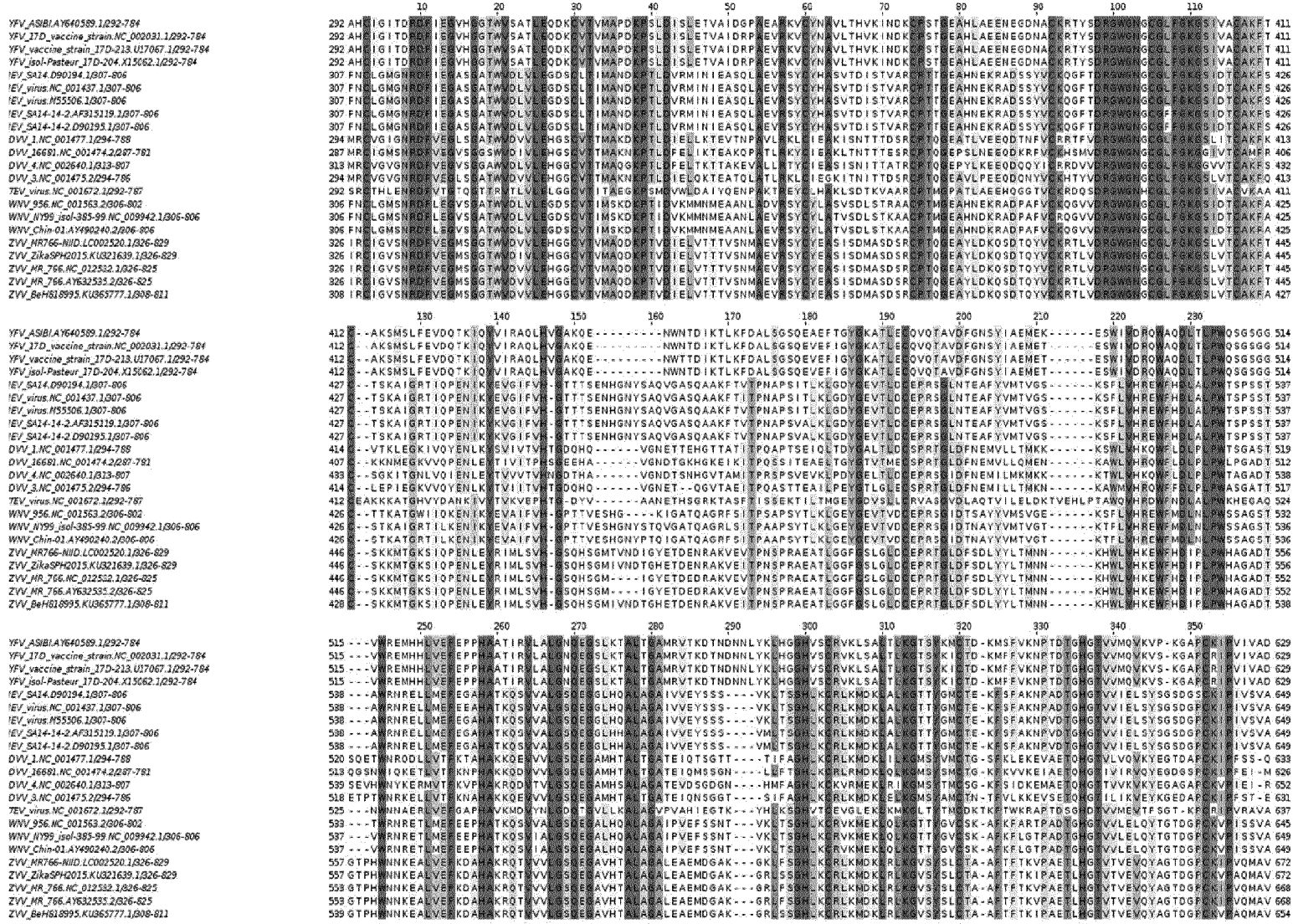
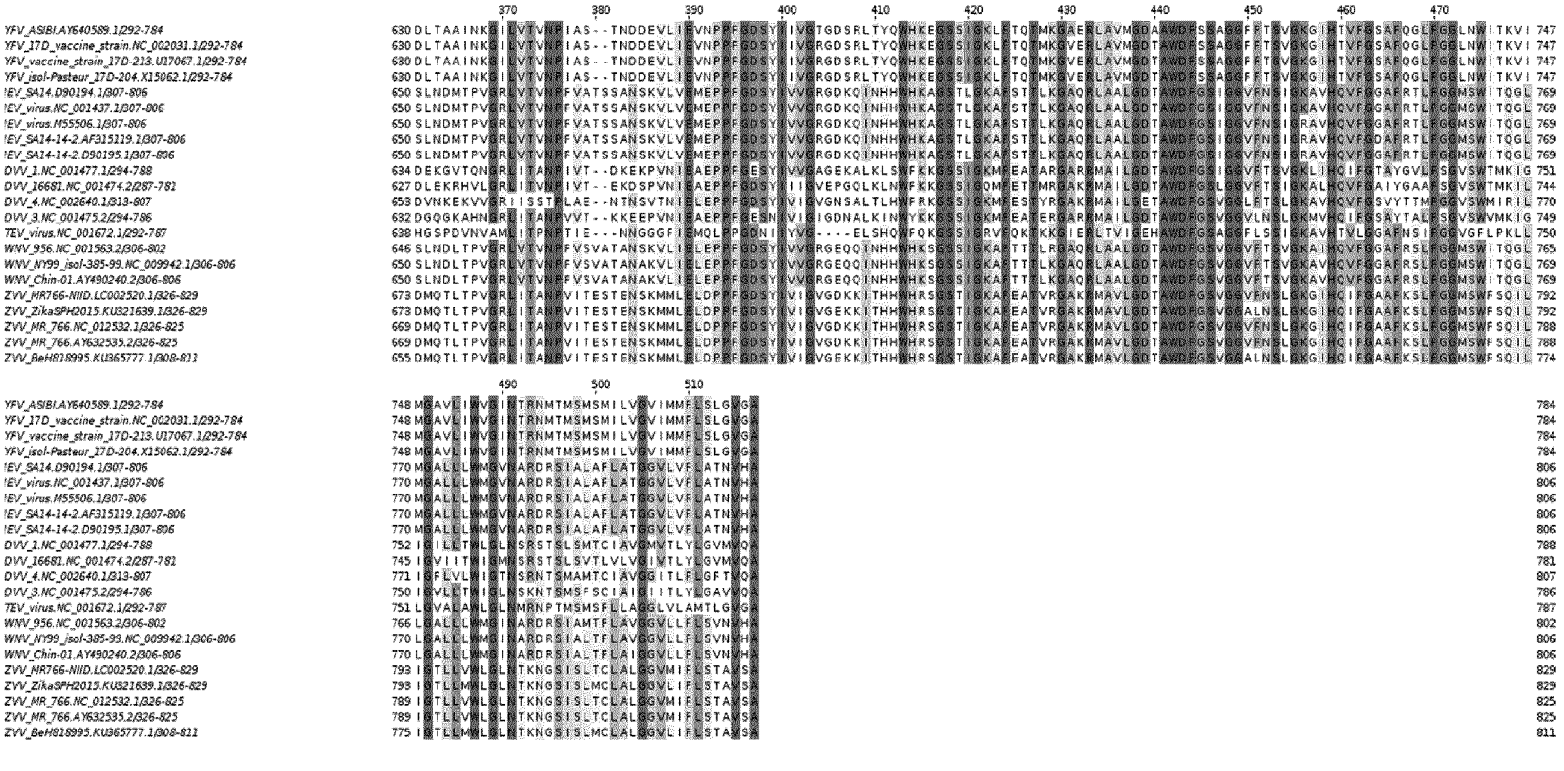

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YFV_17D_vaccine_strain.NC_002031.1/292-784
YFV_vaccine_strain_17D-213.U17067.1/292-784
YFV_isol-Pasteur_17D-204.X15062.1/292-784
!EV_SA14.D90194.1/307-806
!EV_virus.NC_001437.1/307-806
!EV_virus.M55506.1/307-806
!EV_SA14-14-2:AF315119.1/307-806
!EV_SA14-14-2.D90195.1/307-806
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
ZVV_MR766-NIID.LC002520.1/326-829
ZVV_ZikaSPH2015.KU321639.1/326-829
ZVV_MR_766.NC_012532.1/326-825
ZVV_MR_766.AY632535.2/326-525
ZVV_BeH818995.KU365777.1/308-811

Figure 7 (b)


370 380 390 400 410 420 430 440 450 460 470
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
!EV_SA14.D90194.1/307-806
!EV_virus.NC_001437.1/307-806
!EV_virus.M55506.1/307-806
!EV_SA14-14-2.AF315119.1/307-806
!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
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
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
!EV_SA14.D90194.1/307-806
!EV_virus.NC_001437.1/307-806
!EV_virus.M55506.1/307-806
!EV_SA14-14-2.AF315119.1/307-806
!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
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

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Bescheinigung	Certificate	Attestation
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Patentanmeldung Nr.	**Patent application No.**	**Demande de brevet n°**
	16182845.4 / EP16182845	

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP16182845.

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
p.o.

V. Joseph

Anmeldung Nr:
Application no.: 16182845.4
Demande no :

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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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Si aucun titre n'est indiqué, ou si la demande a été déposée dans une langue autre qu'une langue officielle de l'OEB, se référer à la description pour le titre original.)

VIRUS PURIFICATION

In Anspruch genommene Priorität(en) / Priority(Priorities) claimed / Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

Am Anmeldetag benannte Vertragstaaten / Contracting States designated at date of filing / Etats contractants désignés lors du dépôt:

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

PAT018/EP/PR3

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).

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
AGTCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGA
GAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGA
CACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCC
GAAGCCACCCTGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTT
TCAGATTTGTATTACTTGACTATGAATAACAAGCACTGGTTGGTTCACAAGGAGTGGTTCCACGAC
ATTCCATTACCTTGGCACGCTGGGGCAGACACCGGAACTCCACACTGGAACAACAAAGAAGCACT
GGTAGAGTTCAAGGACGCACATGCCAAAAGGCAAACTGTCGTGGTTCTAGGGAGTCAAGAAGGAG
CAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCC
TCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTG
TGTACCGCAGCGTTCACATTCACCAAGATCCCGGCTGAAACACTGCACGGGACAGTCACAGTGGA
GGTACAGTACGCAGGGACAGATGGACCTTGCAAGGTTCCAGCTCAGATGGCGGTGGACATGCAAA
CTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTA
AGATGATGCTGGAACTTGATCCACCATTTGGGGACTCTTACATTGTCATAGGAGTCGGGGAGAAG
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KU501215.1 Zika virus strain PRVABC59, Puerto Rico, complete genome (SEQ ID NO: 4)

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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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AY632535.2 NC_012532.1 Zika virus strain MR 766, Uganda, complete genome (SEQ ID NO: 12)

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

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CCAGATGACGTCGATTGTTGGTGCAACACGACGTCAACTTGGGTTGTGTACGGAACCTGCCATCAC
AAAAAAGGTGAAGCACGGAGATCTAGAAGAGCTGTGACGCTCCCCTCCCATTCCACTAGGAAGCT
GCAAACGCGGTCGCAAACCTGGTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAA
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CAACGAGCCAAAAAGTCATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGCATCAGGT
GCATAGGAGTCAGCAATAGGGACTTTGTGGAAGGTATGTCAGGTGGGACTTGGGTTGATGTTGTCT
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ATGTCTGCAAAAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGG
AGCCTGGTGACATGCGCTAAGTTTGCATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGA
GAATCTGGAGTACCGGATAATGCTGTCAGTTCATGGCTCCCAGCACAGTGGGATGATCGTTAATGA
CACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTCACCAAGAGCC
GAAGCCACCCTGGGGGGGTTTTGGAAGCCTAGGACTTGATTGTGAACCGAGGACAGGCCTTGACTTT
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CAGTTCACACGGCCCTTGCTGGAGCTCTGGAGGCTGAGATGGATGGTGCAAAGGGAAGGCTGTCC
TCTGGCCACTTGAAATGTCGCCTGAAAATGGATAAACTTAGATTGAAGGGCGTGTCATACTCCTTG
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CTCTGACCCCAGTTGGGAGGTTGATAACCGCTAACCCCGTAATCACTGAAAGCACTGAGAACTCTA
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CGGTCGTTGTGGGATCTGTAAAAAACCCCATGTGGAGAGGTCCACAGAGATTGCCCGTGCCTGTG
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GTGATCTGATCATACCCAAGTCTTTAGCTGGGCCACTCAGCCATCACAATACCAGAGAGGGCTACA
GGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGC
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GTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGTGCTTGTGATTCT
GCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCGATG
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CATGCTGCTGGCCTTGGCCTCGTGTCTTTTGCAAACTGCGATCTCCGCCTTGGAAGGCGACCTGAT
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AACATCACCTTGGCAATCCTGGCTGCTCTGACACCACTGGCCCGGGGCACACTGCTTGTGGCGTGG
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GGTGGGACTGCTGTTGCTCACAAGGAGTGGGAAGCGGAGCTGGCCCCCTAGCGAAGTACTCACAG
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GAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCG
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CAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAATGACTCGTAGACTGCTA
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GATCTGGTGTCATACTGTGGTCCATGGAAGCTAGATGCCGCCTGGGACGGGCACAGCGAGGTGCA
GCTCTTGGCCGTGCCCCCCGGAGAGAGAGCGAGGAACATCCAGACTCTGCCCGGAATATTTAAGA
CAAAGGATGGGGACATTGGAGCGGTTGCGCTGGATTACCCAGCAGGAACTTCAGGATCTCCAATC
CTAGACAAGTGTGGGAGAGTGATAGGACTTTATGGCAATGGGGTCGTGATCAAAAATGGGAGTTA
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CATGACCGCCACGCCACCAGGAACCCGTGACGCATTTCCGGACTCCAACTCACCAATTATGGACAC
CGAAGTGGAAGTCCCAGAGAGAGCCTGGAGCTCAGGCTTTGATTGGGTGACGGATCATTCTGGAA
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TGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACC
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TGTGCCGGCAGAGGTGTGGACCAGACACGGAGAGAAAAGAGTGCTCAAACCGAGGTGGATGGAC
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GTTGTGTTCCTATTGCTGGTGGTGCTCATACCTGAGCCAGAAAAGCAAAGATCTCCCCAGGACAAC
CAAATGGCAATCATCATCATGGTAGCAGTAGGTCTTCTGGGCTTGATTACCGCCAATGAACTCGGA
TGGTTGGAGAGAACAAAGAGTGACCTAAGCCATCTAATGGGAAGGAGAGAGGAGGGGGCAACCA
TAGGATTCTCAATGGACATTGACCTGCGGCCAGCCTCAGCTTGGGCCATCTATGCTGCCTTGACAA
CTTTCATTACCCCAGCCGTCCAACATGCAGTGACCACTTCATACAACAACTACTCCTTAATGGCGA

TGGCCACGCAAGCTGGAGTGTTGTTTGGTATGGGCAAAGGGATGCCATTCTACGCATGGGACTTTG
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TTTTGCTCGTGGCGCACTACATGTACTTGATCCCAGGGCTGCAGGCAGCAGCTGCGCGTGCTGCCC
AGAAGAGAACGGCAGCTGGCATCATGAAGAACCCTGTTGTGGATGGAATAGTGGTGACTGACATT
GACACAATGACAATTGACCCCCAAGTGGAGAAAAAGATGGGACAGGTGCTACTCATAGCAGTAGC
CGTCTCCAGCGCCATACTGTCGCGGACCGCCTGGGGGGTGGGGGGGAGGCTGGGGCCCTGATCACAG
CGGCAACTTCCACTTTGTGGGAAGGCTCTCCGAACAAGTACTGGAACTCCTCTACAGCCACTTCAC
TGTGTAACATTTTTAGGGGAAGTTACTTGGCTGGAGCTTCTCTAATCTACACAGTAACAAGAAACG
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CTTGAACCAGATGTCGGCCCTGGAGTTCTACTCCTACAAAAAGTCAGGCATCACCGAGGTGTGCAG
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CACTATGATGGAAACCCTGGAGCGACTGCAGCGTAGGTATGGGGGAGGACTGGTCAGAGTGCCAC
TCTCCCGCAACTCTACACATGAGATGTACTGGGTCTCTGGAGCGAAAAGCAACACCATAAAAAGT
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AGATCATTGGTAACCGCATTGAAAGGATCCGCAGTGAGCACGCGGAAACGTGGTTCTTTGACGAG
AACCACCCATATAGGACATGGGCTTACCATGGAAGCTATGAGGCCCCCACACAAGGGTCAGCGTC
CTCTCTAATAAACGGGGTTGTCAGGCTCCTGTCAAAACCCTGGGATGTGGTGACTGGAGTCACAGG
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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)

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)

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 ≤ 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 ≤ 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

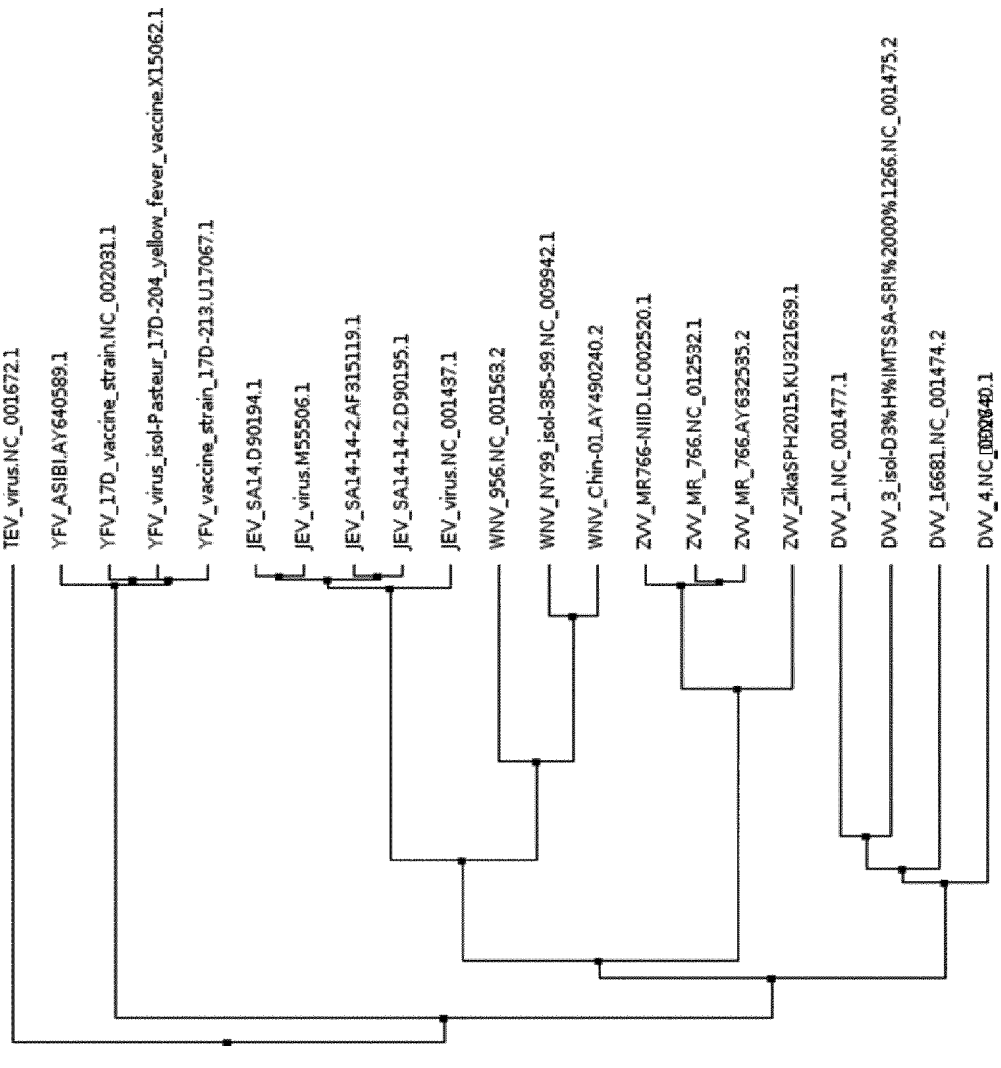

TEV_virus.NC_001672.1
YFV_ASIB1.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_virus_isol-Pasteur_17D-204_yellow_fever_vaccine.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
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

Figure 2

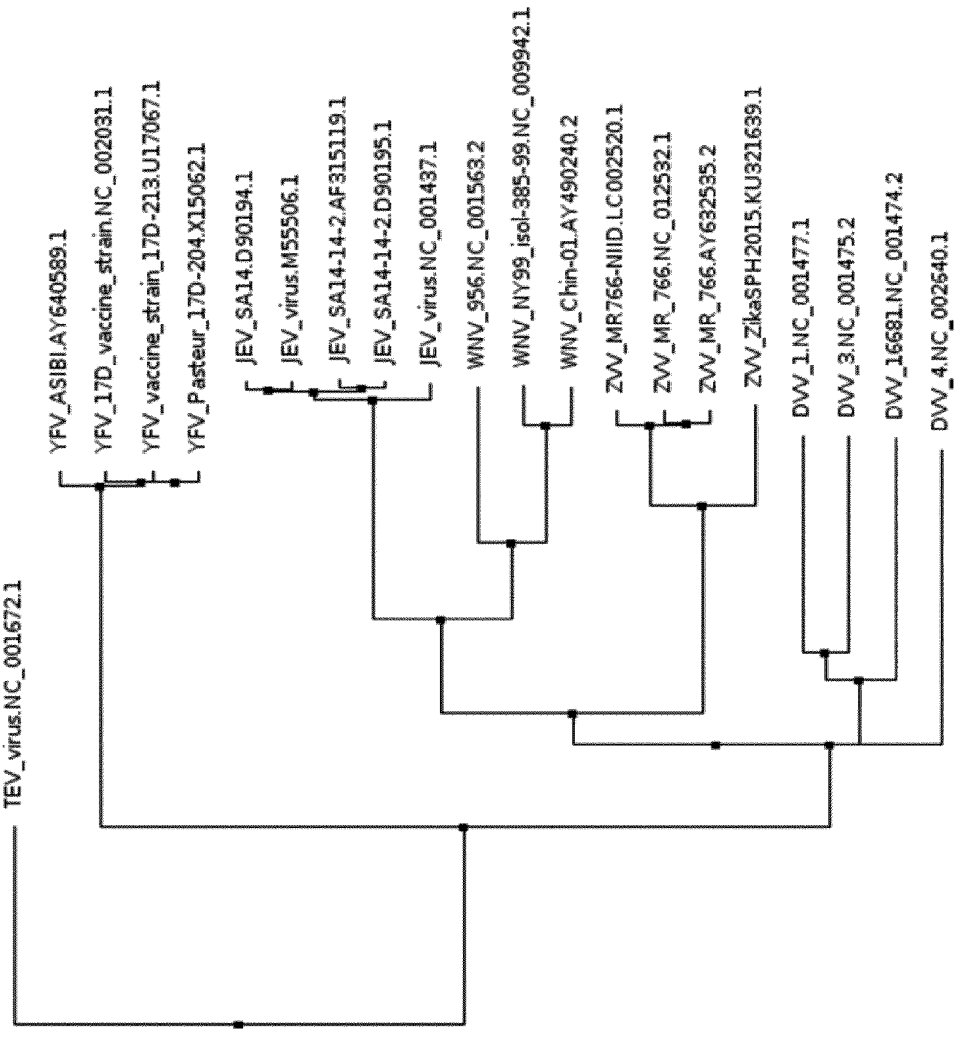

TEV_virus.NC_001672.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_vaccine_strain_17D-213.U17067.1
YFV_Pasteur_17D-204.X15062.1
JEV_SA14.D90194.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
JEV_virus.NC_001437.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
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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

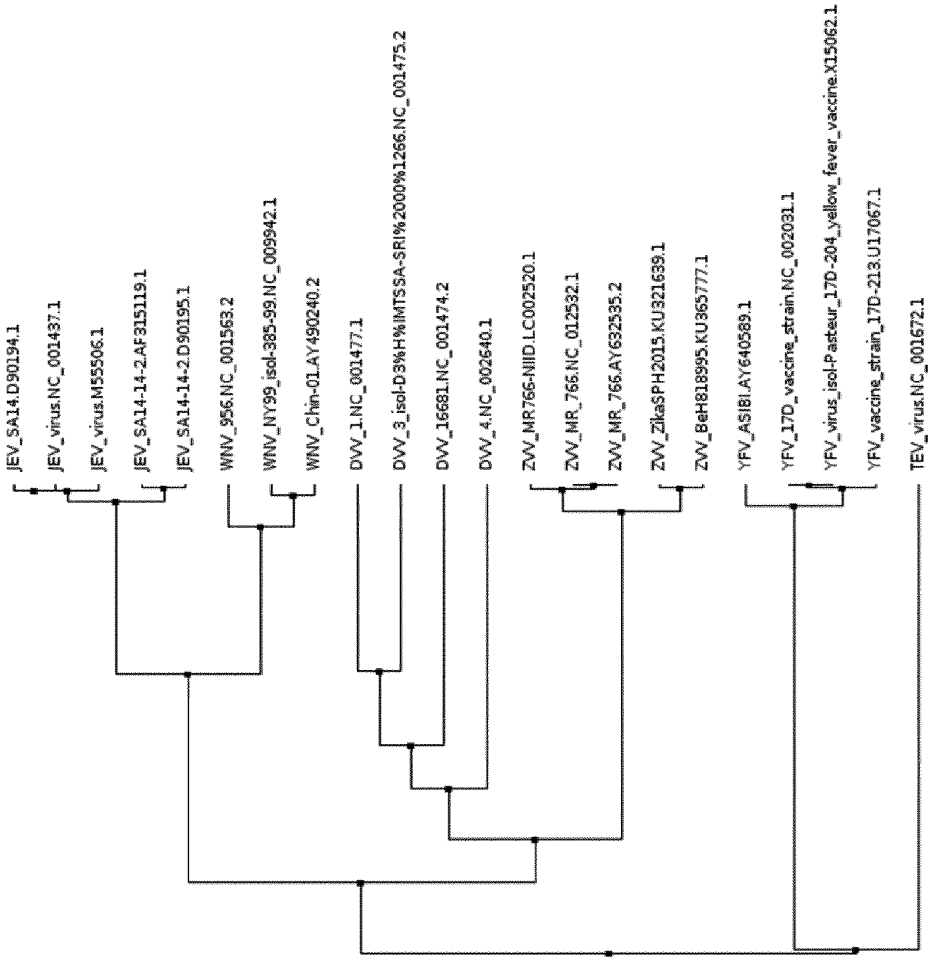

JEV_SA14.D90194.1
JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
WNV_956.NC_001563.2
WNV_NY99_isol-385-99.NC_009942.1
WNV_Chin-01.AY490240.2
DVV_1.NC_001477.1
DVV_3_isol-D3%H%IMTSSA-SRI%2000%1266.NC_001475.2
DVV_16681.NC_001474.2
DVV_4.NC_002640.1
ZVV_MR766-NIID.LC002520.1
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 5

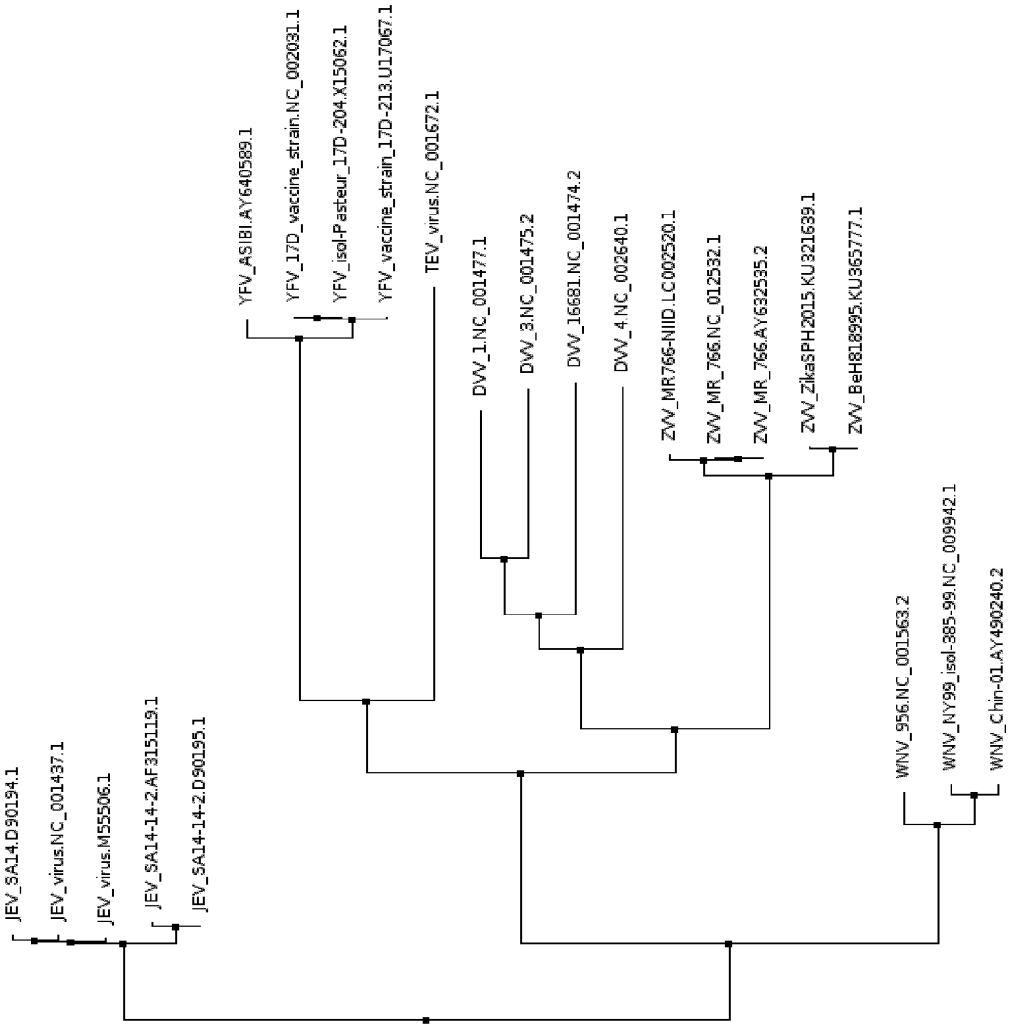

JEV_SA14.D90194.1
JEV_virus.NC_001437.1
JEV_virus.M55506.1
JEV_SA14-14-2.AF315119.1
JEV_SA14-14-2.D90195.1
YFV_ASIBI.AY640589.1
YFV_17D_vaccine_strain.NC_002031.1
YFV_isol-Pasteur_17D-204.X15062.1
YFV_vaccine_strain_17D-213.U17067.1
TEV_virus.NC_001672.1
DVV_1.NC_001477.1
DVV_3.NC_001475.2
DVV_16681.NC_001474.2
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

699

Figure 7 (a)

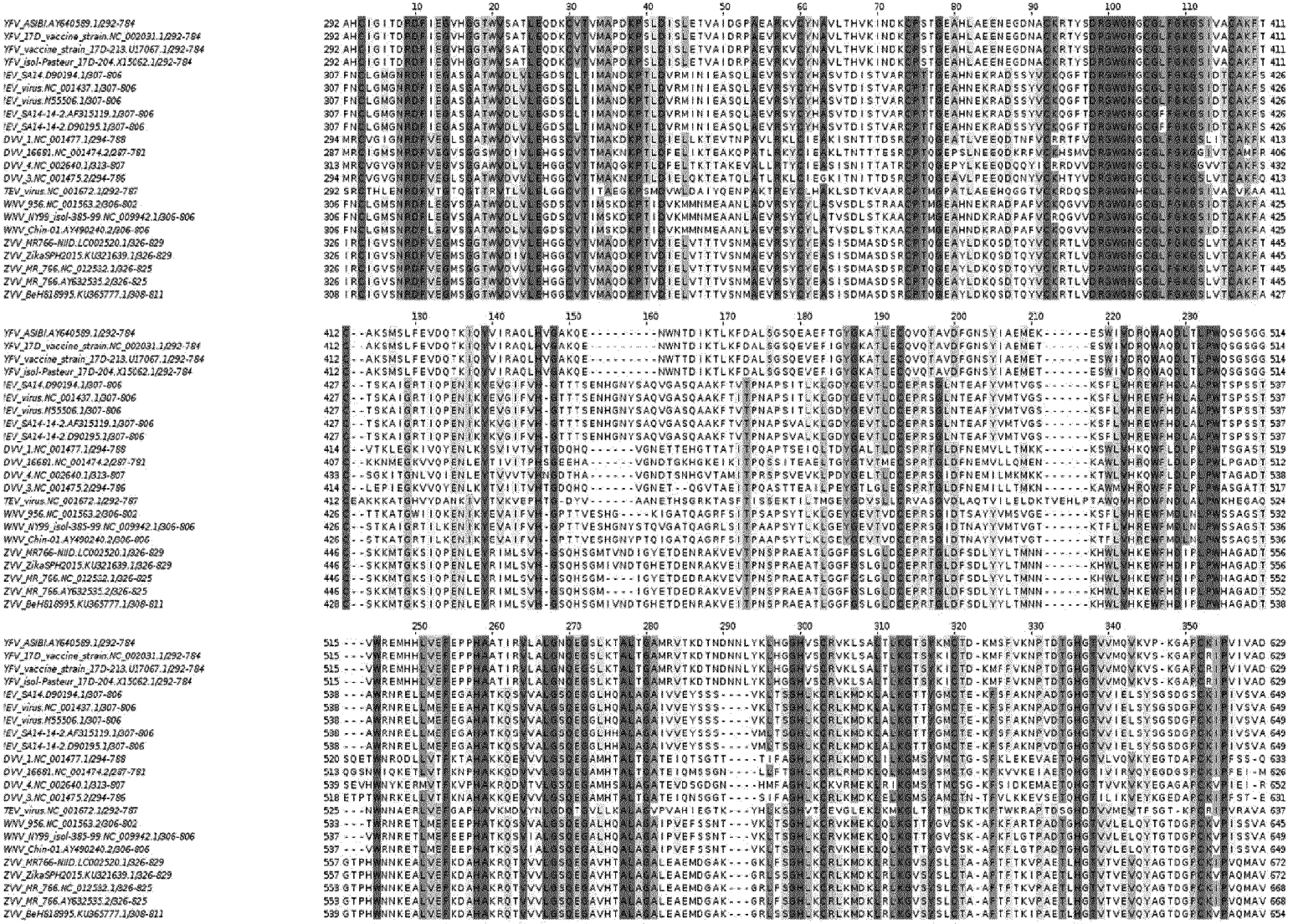

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DVV_4.NC_002640.1/313-807
DVV_3.NC_001475.2/294-786
TEV_virus.NC_001672.1/292-787
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Figure 7 (b)

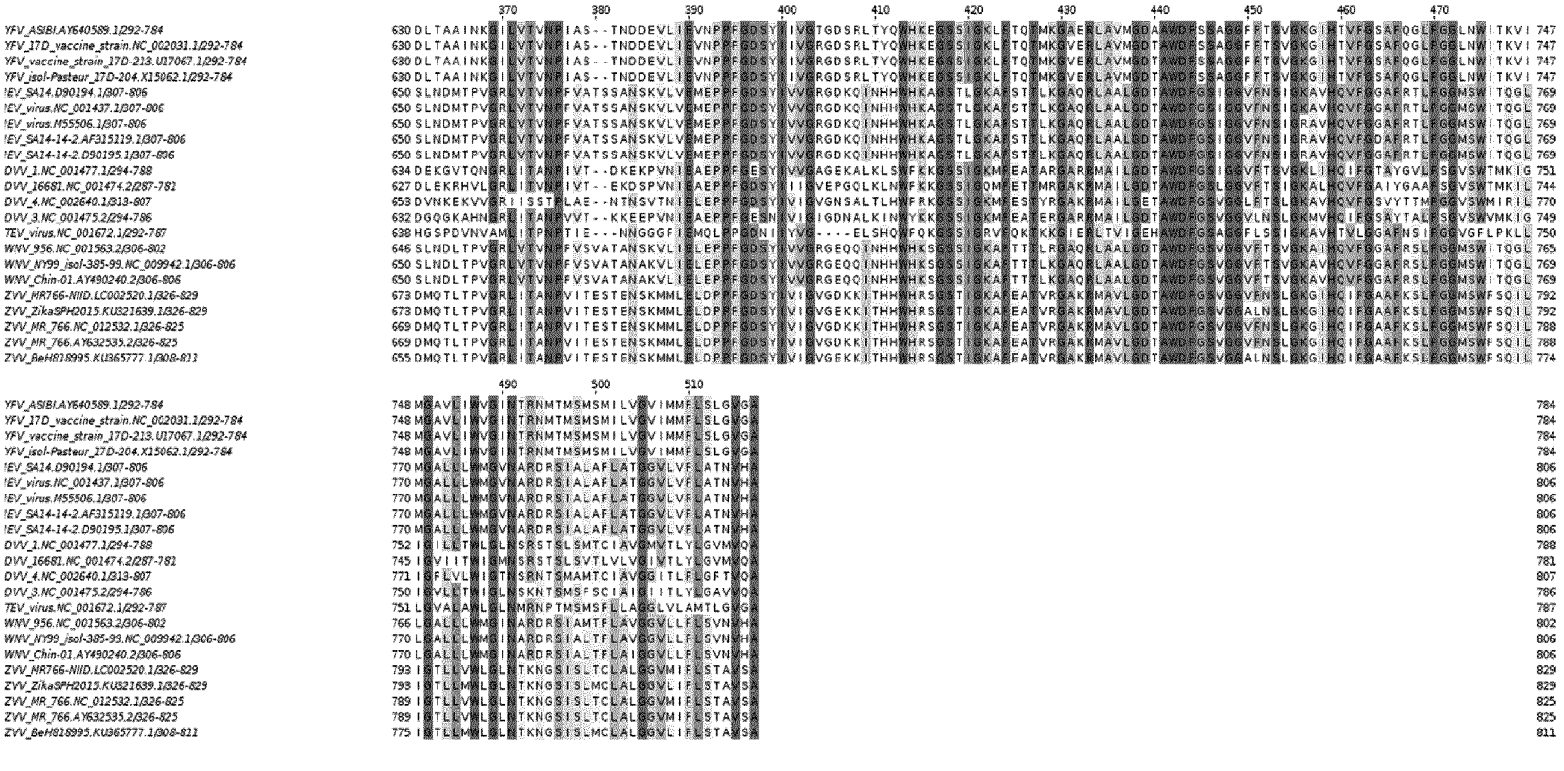

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DVV_4.NC_002640.1/313-807
DVV_3.NC_001475.2/294-786
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WNV_Chin-01.AY490240.2/306-806
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490 500 510
784
802
806
829
825
811

Figure 8a

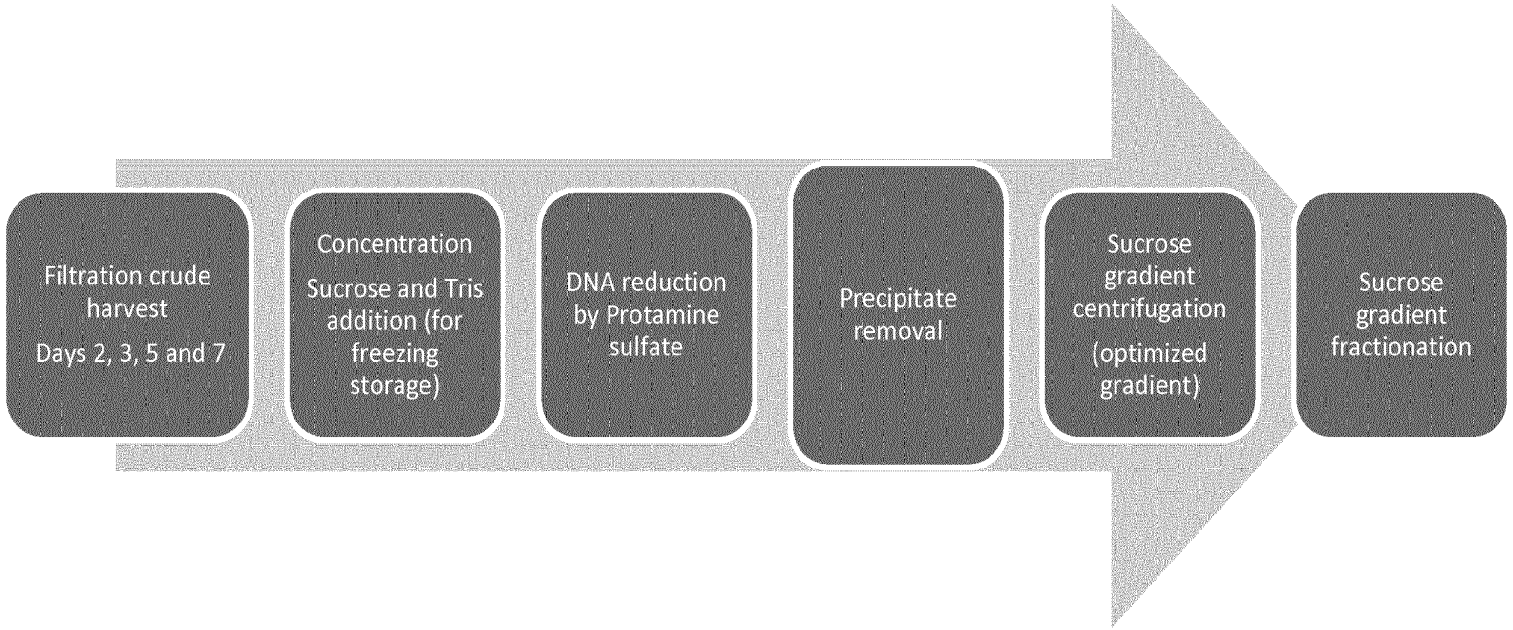

Filtration crude harvest
Days 2, 3, 5 and 7
Concentration
Sucrose and Tris addition (for freezing storage)
DNA reduction by Protamine sulfate
Precipitate removal
Sucrose gradient centrifugation
(optimized gradient)
Sucrose gradient fractionation

Figure 8b

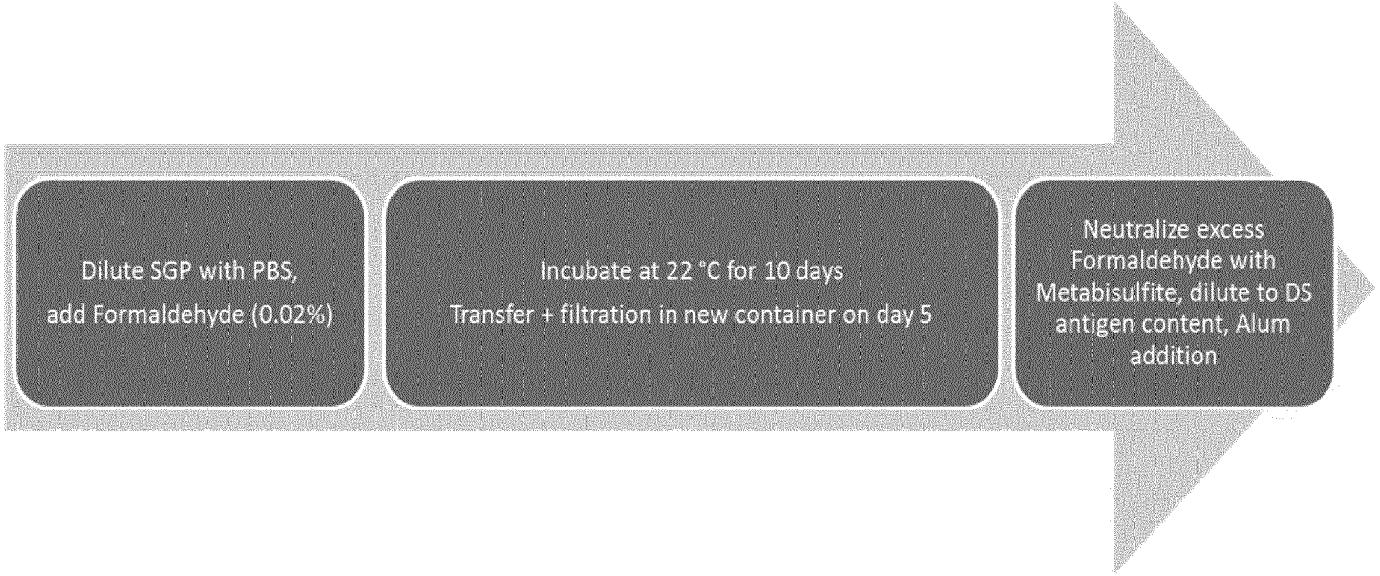
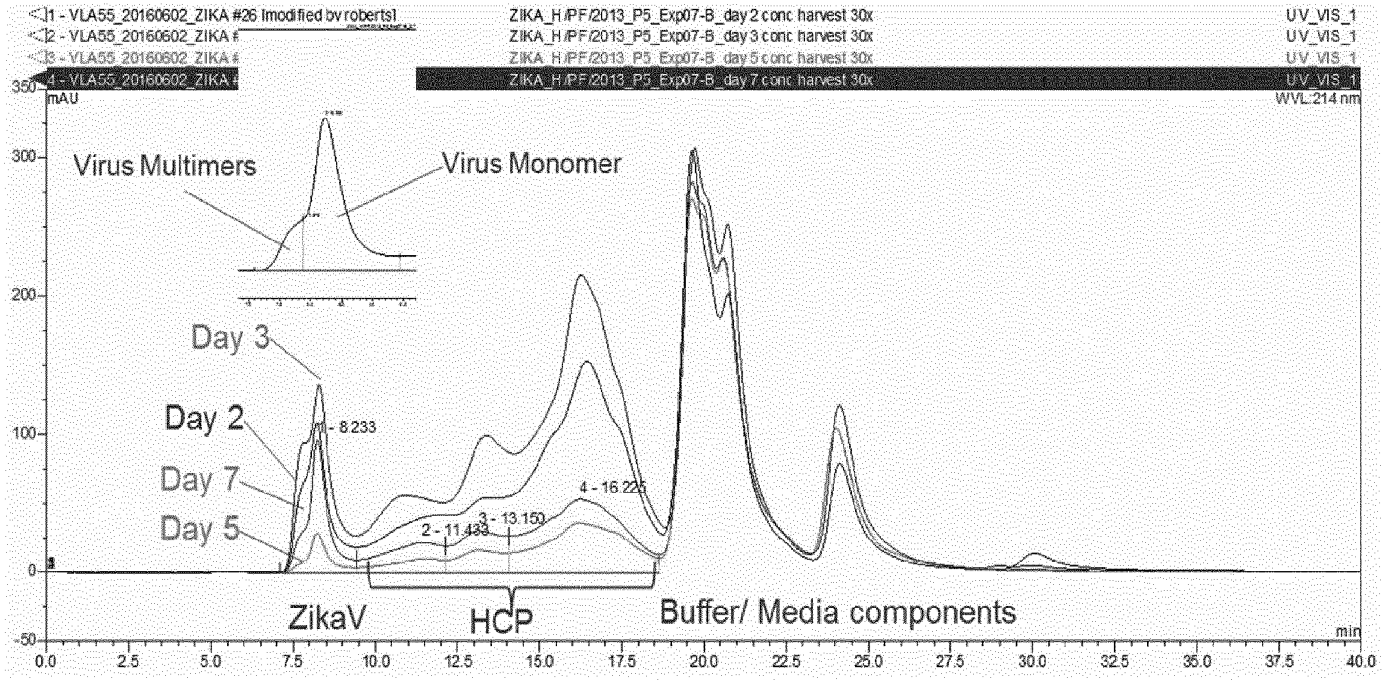
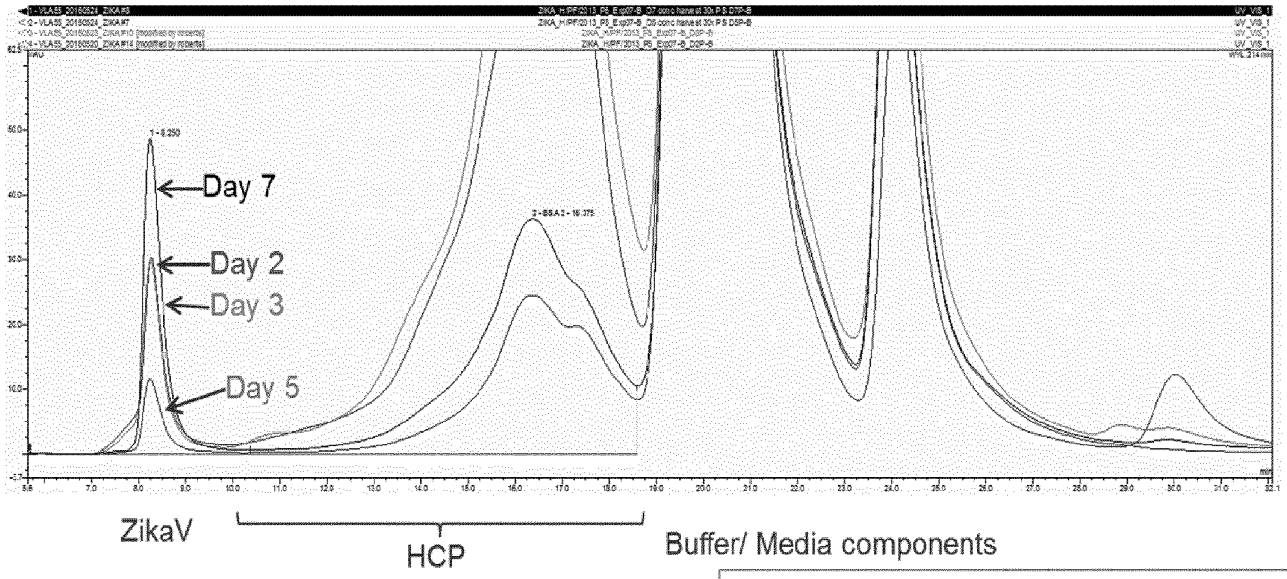

Dilute SGP with PBS,
add Formaldehyde (0.02%)
Incubate at 22 °C for 10 days
Transfer + filtration in new container on day 5
Neutralize excess Formaldehyde with Metabisulfite, dilute to DS antigen content, Alum addition

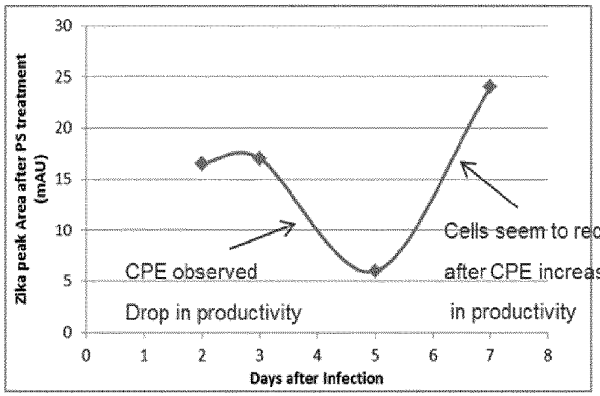
Figure 9



+ 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

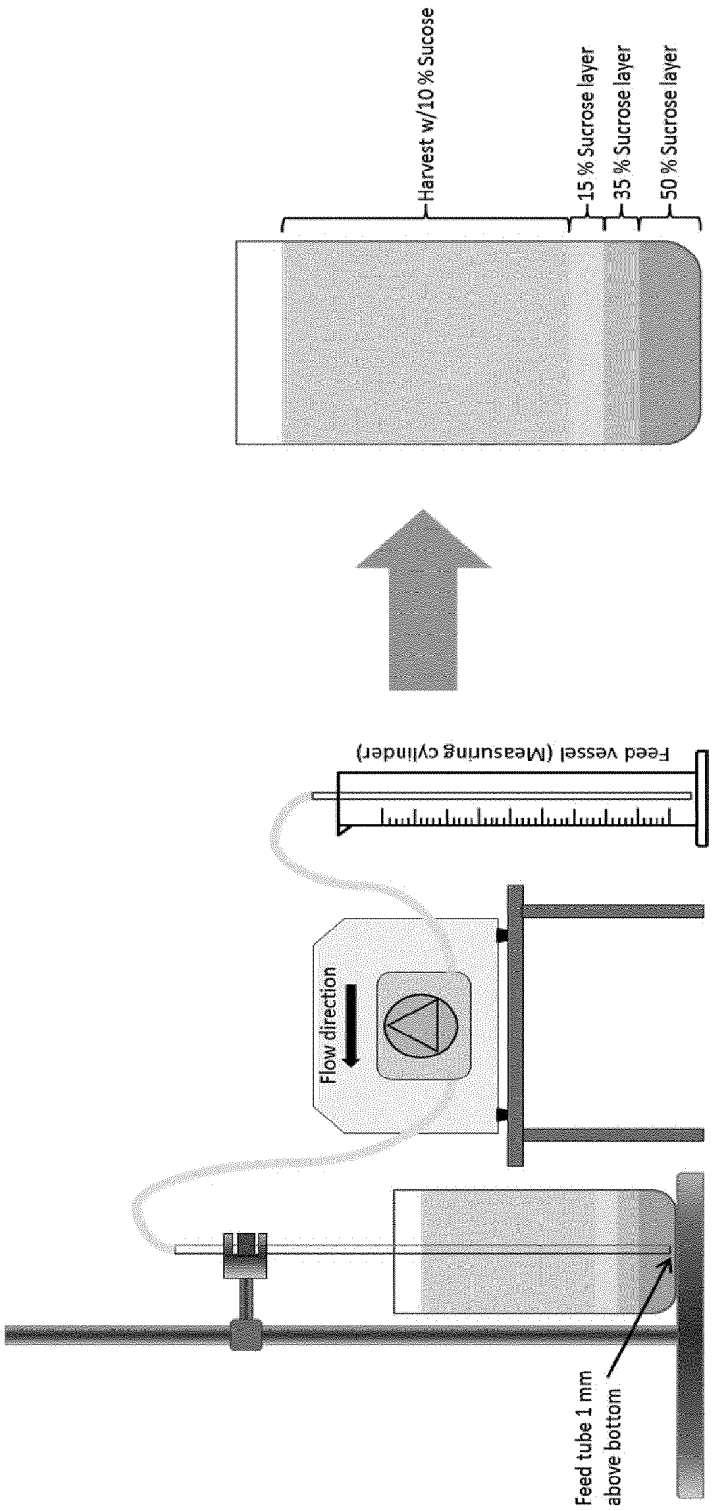
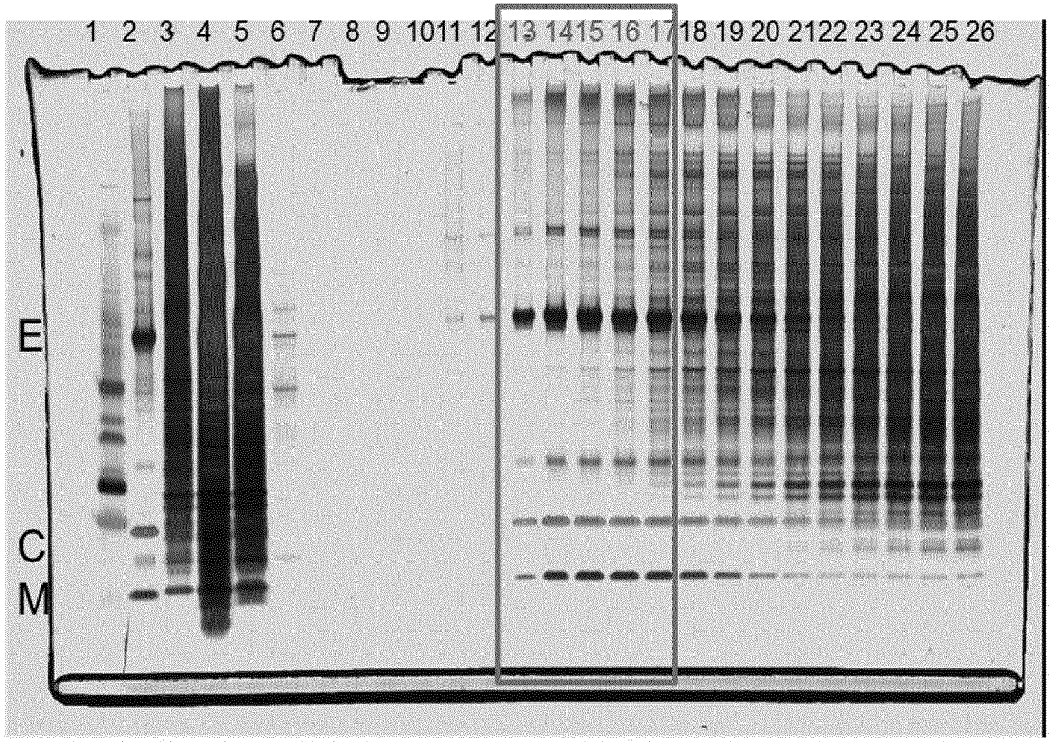

Harvest w/10 % Sucose
15 % Sucrose layer
35 % Sucrose layer
50 % Sucrose layer
Feed vessel (Measuring cylinder)
Flow direction
Feed tube 1 mm above bottom

Figure 12

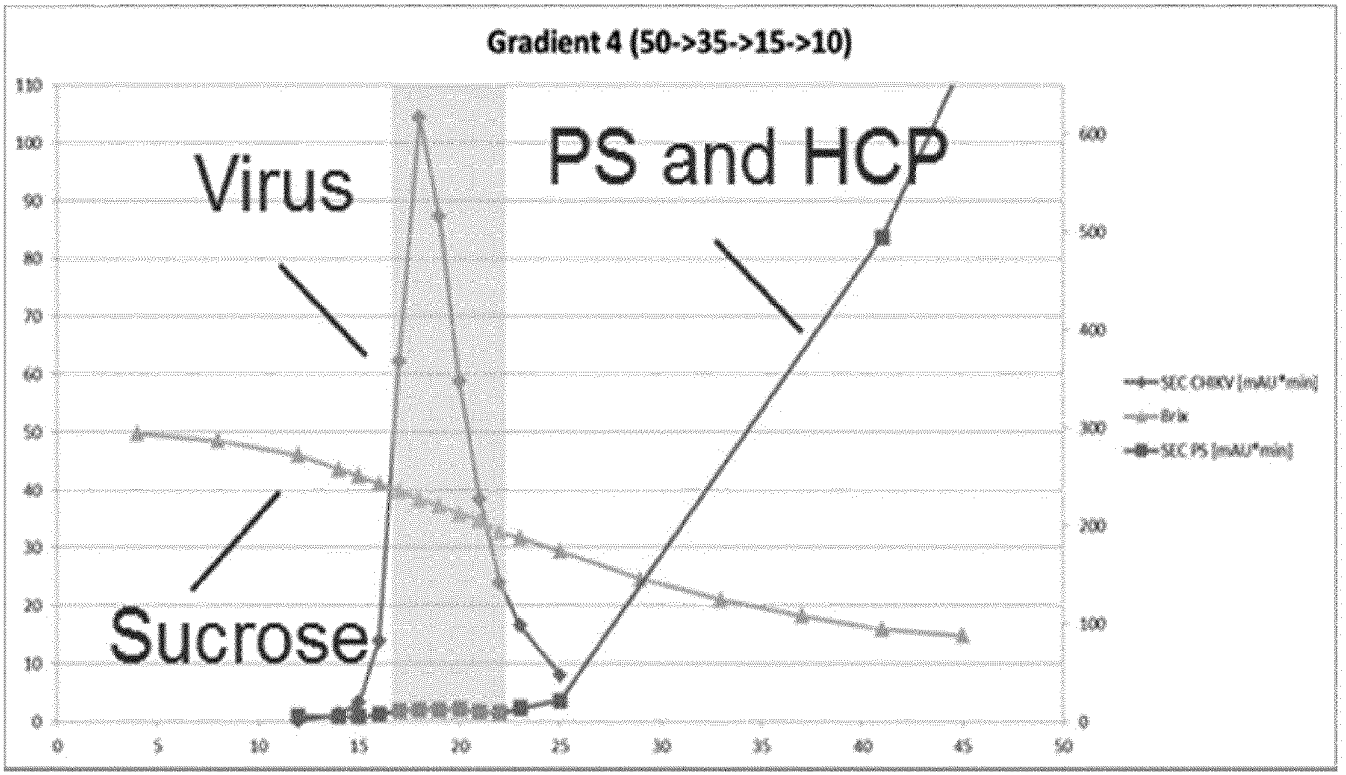


Pooled fractions F10-F14
(10mL total volume per bottle)

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



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Figure 14

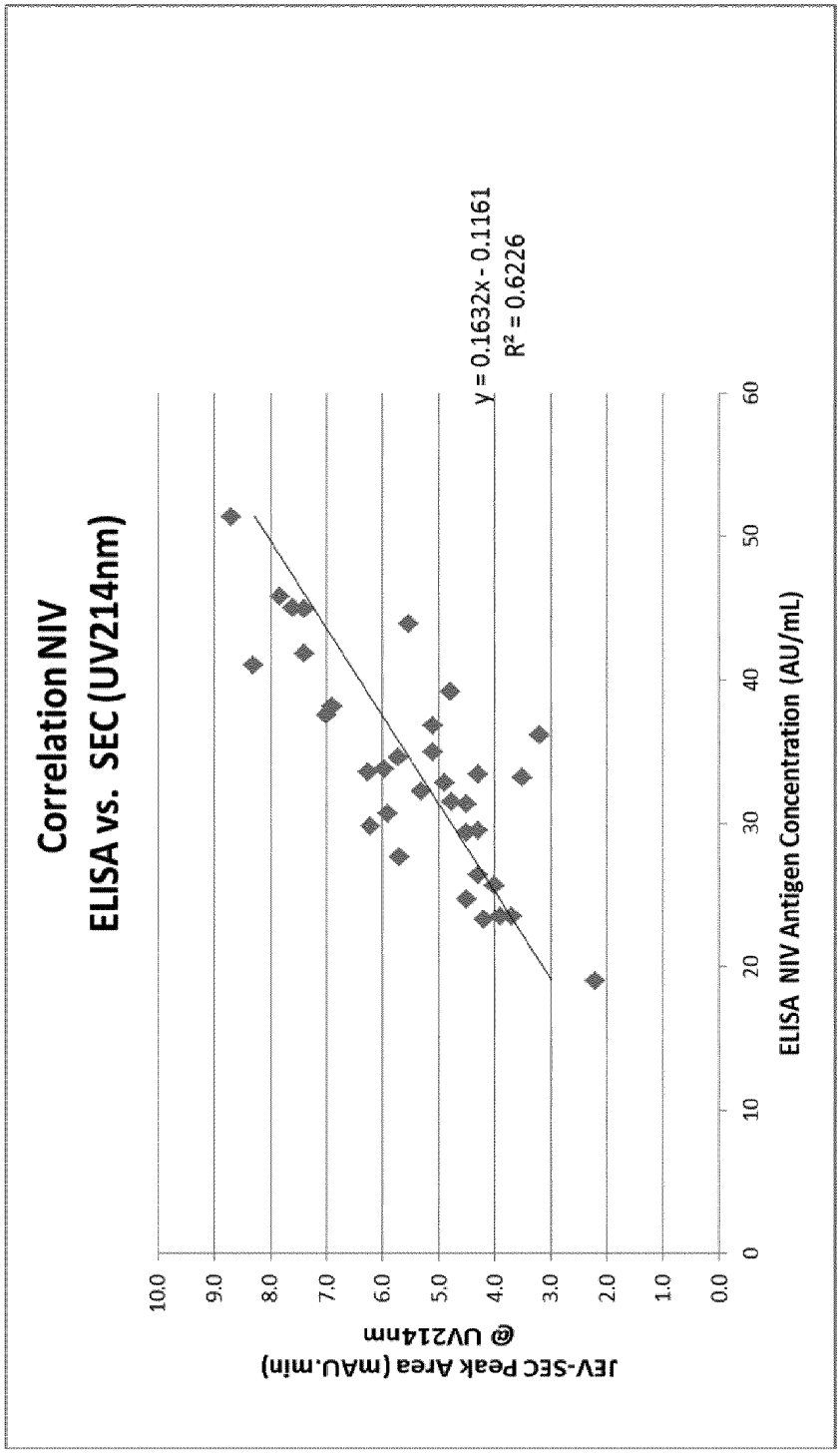
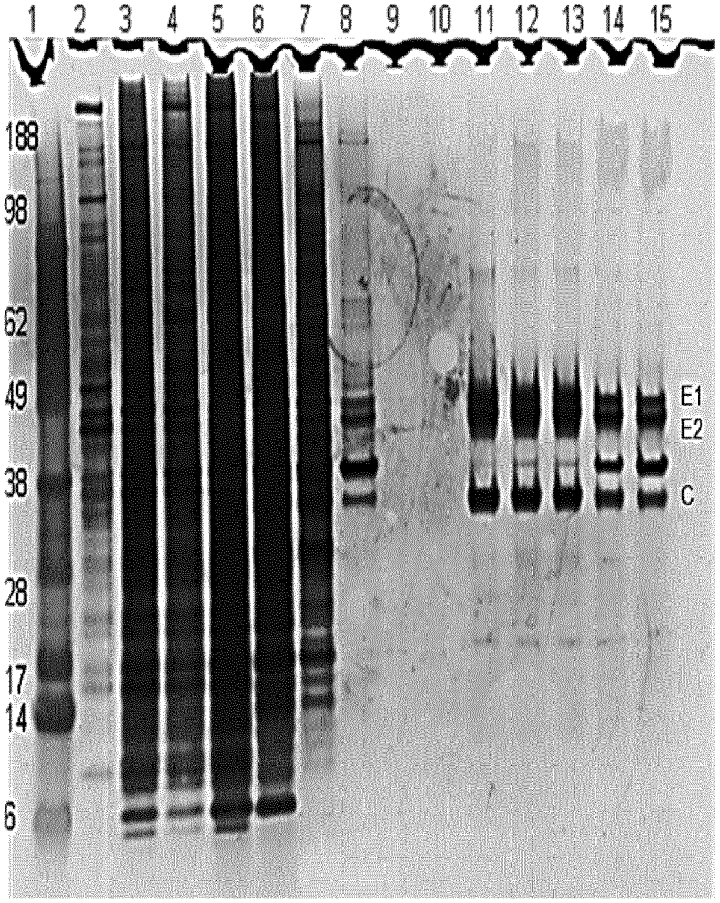
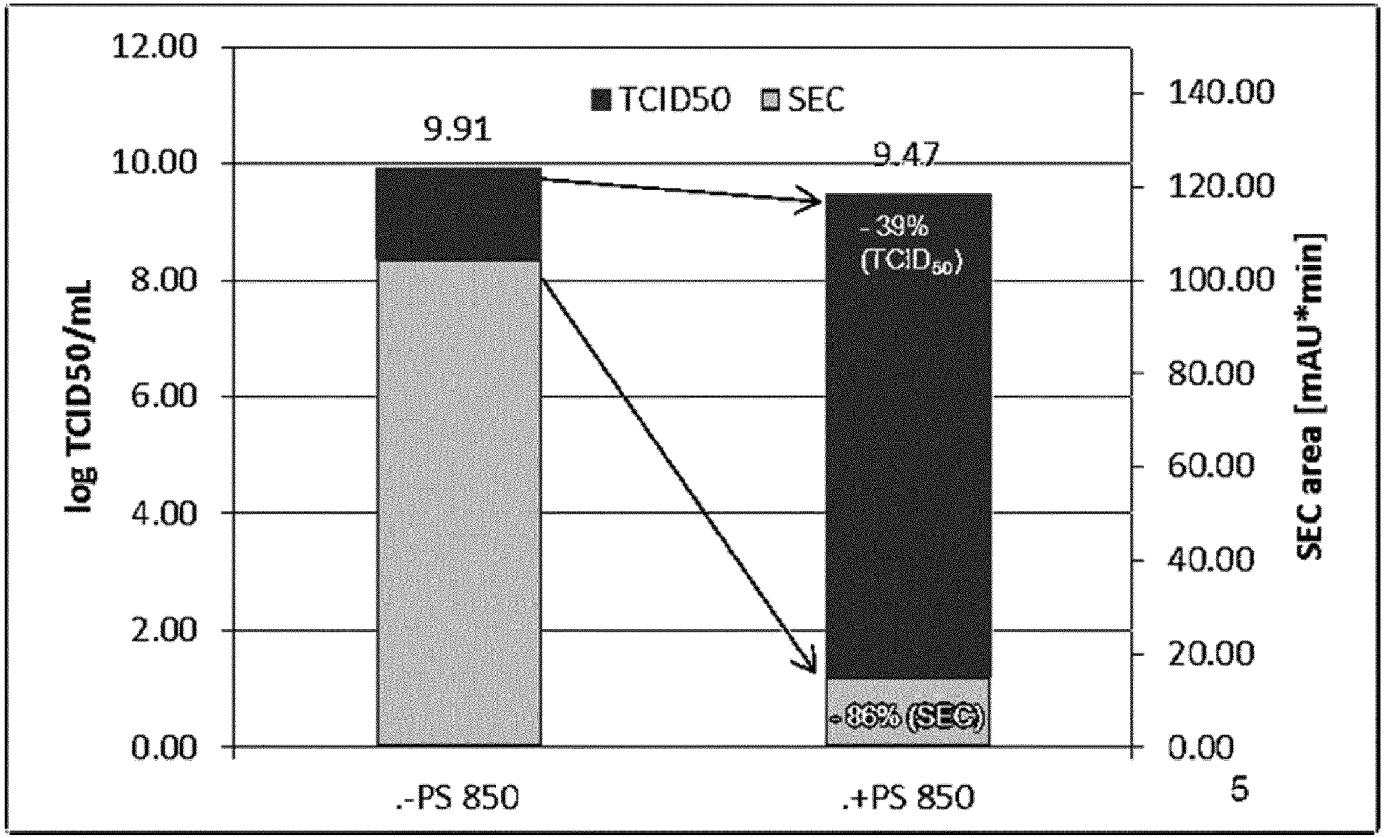

Correlation NIV
ELISA vs. SEC (UV214nm)
y = 0.1632x - 0.1161
R² = 0.6226
JEV-SEC Peak Area (mAU.min)
@ UV214nm
ELISA NIV Antigen Concentration (AU/mL)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
10
20
30
40
50
60

Figure 15



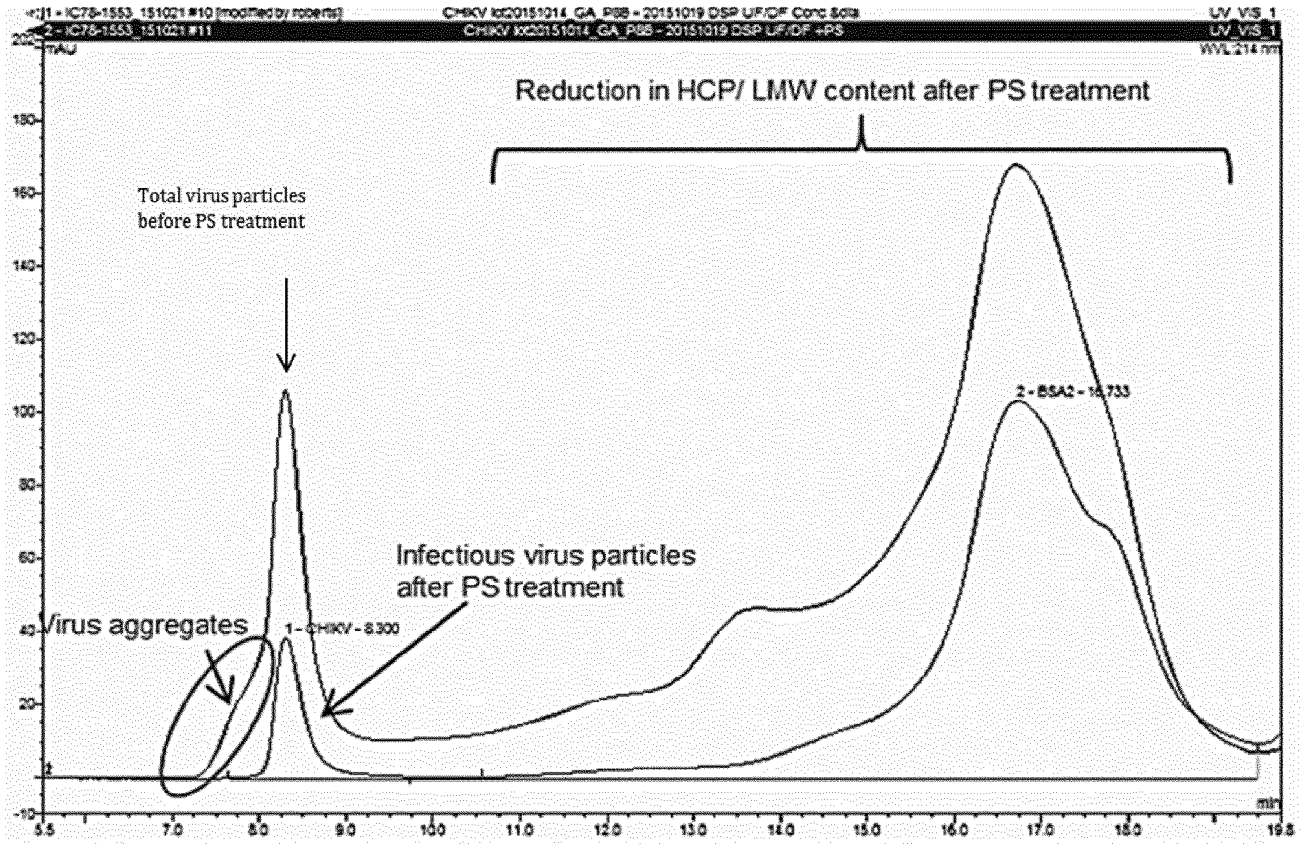
Lane	Sample
1	Marker Seeblueplus2
2	CHIKV lot20150812_WVB2015-01_Filtered harvest 0.2µ_24hpi
3	CHIKV lot20150812_WVB2015-01_Filtered harvest 0.2µ_48hpi
4	20150813_CHIKV_DSP_UF/DF_Load
5	20150813_CHIKV_DSP_UF/DF_conc. 10x
6	20150813_CHIKV_DSP_UF/DF_conc.&dia. 11x
7	20150813_CHIKV_DSP_PStreatment
8	20150813_CHIKV_DSP_PS&CC700treatment
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



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Figure 16b



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