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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371

Attorney Docket No.	
159792010300	
U.S. Application No. (if known, see 37 CFR 1.5)	
Not Yet Assigned	

International Application No.	International Filing Date	Priority Date Claimed
PCT/US2016/013947	19 January 2016	20 January 2015

Title of Invention: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

First Named Inventor: Catherine R. O'RIORDAN

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

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

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

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

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

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

PCT Article 19 and 34 amendments

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

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

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

Cancellation of amendments made in the international phase

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

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

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

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

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

10. [x] A preliminary amendment.

11. [x] An Application Data Sheet under 37 CFR 1.76.

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

13. [x] A power of attorney and/or change of address letter.

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

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

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

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U.S. APPLN. No. (if known – see 37 CFR 1.5)	INTERNATIONAL APPLICATION No.	ATTORNEY DOCKET No.
Not Yet Assigned	PCT/US2016/013947	159792010300

17. ☐ Other items or information:

The following fees have been submitted.	CALCULATIONS	PTO USE ONLY
18. ☒ Basic national fee (37 CFR 1.492(a)) **$280**	$ 280.00	
19. ☒ Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... **$0** All other situations **$720**	$ 720.00	
20. ☒ Search fee (37 CFR 1.492(b)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... **$0** Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority ..... **$120** International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB ..... **$480** All other situations **$600**	$ 480.00	
TOTAL OF 18, 19, and 20 =	$ 1,480.00	
☐ Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) in an electronic medium or computer program listing in an electronic medium) (37 CFR 1.492(j)). Fee for each additional 50 sheets of paper or fraction thereof **$400**		
Total Sheets: 105- 100 = / Extra Sheets: 1 / 50 = / Number of each addition 50 or fraction thereof (round **up** to a whole number): 1 / RATE: x **$400**	$ 400.00	
Surcharge of **$140.00** for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h)).	$ 0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =	0	x **$80**	$ 0.00	
Independent claims	- 3 =	0	x **$420**	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$780**	$ 0.00	
Processing fee of **$140.00** for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i)). +				$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 1,880.00	
☐ **Applicant asserts small entity status.** See 37 CFR 1.27. Fees above are reduced by ½.					
☐ **Applicant certifies micro entity status.** See 37 CFR 1.29. Fees above are reduced by ¾. Applicant must attach form PTO/SB/15A or B or equivalent.					
TOTAL NATIONAL FEE =				$	1,880.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +				$	0.00
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				Amount to be refunded:	$
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a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 03-1952 in the amount of $ 1,880.00 to cover the above fees.

c. [X] The Director is hereby authorized to charge additional fees which may be required, or credit any overpayment, to Deposit Account No. 03-1952 as follows:

i. [ ] any required fee.

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

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

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

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

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

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

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Signature	/Brian A. Donahue/	Date	July 18, 2017
Name (Print/Type)	Brian A. Donahue	Registration No. (Attorney/Agent)	58,206

I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: July 18, 2017 Electronic Signature for Ruth Lang /Ruth Lang/

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

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(54) Title: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

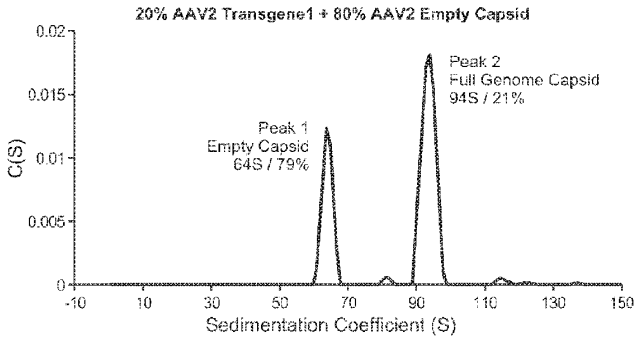


FIG. 1B

(57) Abstract: Provided herein are methods to characterize preparations of recombinant viral particles using analytical ultracentrifugation. Recombinant viral particles include recombinant adeno- associated viral particles, recombinant adenoviral particles, recombinant lentiviral particles and recombinant herpes simplex virus particles. Variant species of recombinant viral particles including empty capsids and recombinant viral particles with variant genomes (e.g., truncated genomes, aggregates, recombinants) can be identified and quantitated. The methods can be used to characterize preparations of recombinant viral particles regardless of the sequence of the recombinant viral genome or the serotype of the recombinant viral capsid.

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ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/105,714, filed on January 20, 2015, the content of which is hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to methods to characterize recombinant viral vectors; *e.g.*, recombinant adeno-associated viral (AAV) particles, recombinant adenoviral (rAd) particles, recombinant lentiviral particles and recombinant Herpes simplex viral (rHSV) particles using analytical ultracentrifugation.

BACKGROUND OF THE INVENTION

[0003] Recombinant viruses show great promise and utility as a vehicle to deliver therapeutic nucleic acids for gene therapy applications. A number of different recombinant viruses are used in these gene therapy applications based on a number of factors including the size of the nucleic acid to be delivered, the target cell or tissue to deliver the nucleic acid, the need for short or long term expression of the therapeutic nucleic acid, and integration of the therapeutic nucleic acid into the recipient's genome. Examples of viruses used in gene therapy applications include adeno-associated virus (AAV), adenovirus, lentivirus and herpes simplex virus (HSV).

[0004] The generation of recombinant viral vectors for the clinic requires an analytical method that monitors drug product quality with regard to homogeneity, purity and consistency of manufacturing, yet to date no method to support such a characterization has been established. Typically, the DNA content of recombinant viral DNA viral vectors is measured by Southern blot analysis using a sequence specific probe. Viral capsids or envelopes may be characterized by immunoassay using an antibody that binds specifically to a capsid or envelope protein of a particular recombinant virus. For example, Steinbach, S *et al.*, (1997) *J. Gen. Virol.*, 78:1453-

1462 provides an immunoassay for rAAV serotypes. What is needed is a generic assay to characterize recombinant viral preparations regardless of the nucleic acid sequence of the recombinant viral genome or the serotype of the capsid.

[0005] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0006] In some aspects, the invention provides methods of characterizing a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

[0007] In some aspects, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

[0008] In some aspects, the invention provides methods to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of

recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

[0009] In some aspects, the invention provides, methods of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, and d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

[0010] In some aspects, the invention provides methods of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

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[0011] In some aspects, the invention provides methods of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

[0012] In some aspects, the invention provides methods of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

[0013] In some aspects, the invention provides, methods of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method of any one of claims 5-8, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the

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preparation of recombinant viral particles. In some embodiments, the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles. In some embodiments, the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes. In some embodiments, the capsid particles comprising variant sized genomes comprise truncated genomes, aggregates, recombinants and/or DNA impurities.

[0014] In some aspects, the invention provides methods of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation. In some embodiments, the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes. In some embodiments, the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles.

[0015] In some aspects, the invention provides methods of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the above method, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

[0016] In some embodiments of the above aspects, sedimentation of recombinant viral particles is monitored by absorbance. In some embodiments, the absorbance is at about 230 nm, 260 nm or 280 nm. In some embodiments, the absorbance is at about 260 nm. In some

embodiments, sedimentation of recombinant viral particles is monitored by interference. In some embodiments, the interference is Rayleigh interference.

[0017] In some embodiments of the above aspects, the preparation is an aqueous solution. In further embodiments, the aqueous solution comprises a pharmaceutical formulation. In some embodiments, the aqueous solution comprises a buffer. In some embodiments, the buffer is at physiological pH. In some embodiments, the buffer is at physiological osmolality. In some embodiments, the pharmaceutical formulation comprises phosphate buffered saline (PBS). In some embodiments, the PBS has pH of about 7.2 and an osmolality of about 300 mOsm/L. In some embodiments, the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

[0018] In some embodiments of the above aspects, the C(S) values are determined by an algorithm that comprises Lamm equation solutions. In some embodiments, the algorithm is the SEDFIT algorithm. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge; for example, the sector may be a portion of the ultracentrifuge comprising a detection system. In some embodiments, the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until recombinant viral particles sediment to the bottom of ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window.

[0019] In some embodiments, the radial concentration is recorded for at least about any of 0.5 hours, 0.75 hours, 1.0 hours, 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, or 5.0 hours. In some embodiments, the radial concentration is recorded for about 1.0 hour. In some embodiments, the radial concentration is recorded for about 1.2 hours. In some embodiments, the radial concentration is recorded from about 0.5 hours to about 2.0 hours. In some embodiments, the radial concentration is recorded from about 1.0 hours to about 2.0 hours.

[0020] In some embodiments of the above aspects, at least 30 scans are used to monitor sedimentation of recombinant viral particles. In some aspects, about 30 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 30 to about 75 scans

are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 30 to about 50 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 50 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

[0021] In some embodiments of the above aspects, a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68. In some embodiments, the regularization is a second derivative regularization. In some embodiments, the regularization is Max entropy regularization. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.90. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.99. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68.

[0022] In some embodiments of the above aspects, the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software. In some embodiments, resolution is about 200S to about 1000S. In some embodiments, resolution is about 200S. In some embodiments, S min is about 1. In some embodiments, Smax is about 100S to about 1000S. In other embodiments, Smax is about 200S to about 5000S. In other embodiments, Smax is about 200S. In some embodiments, the frictional ratio is left to float to a value determined by centrifugation software. In some embodiments, the frictional ratio is about 1.0. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

[0023] In some embodiments of the above aspects, the sedimentation of recombinant viral particles is monitored about every 10-60 seconds. In some embodiments, sedimentation of recombinant viral particles is monitored (*e.g.*, scanned) about every 10 seconds. In other embodiments, the sedimentation of recombinant viral particles is monitored about every 60 seconds. In some embodiments, the sedimentation velocity of recombinant viral during ultracentrifugation is determined by monitoring the sedimentation of recombinant viral particles once in more than about every 15 seconds, 30 seconds, 45 seconds, 1 minute (60 seconds), 2

minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes.

[0024] In some embodiments of the above aspects, the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm. In some embodiments, the boundary sedimentation velocity is performed at about 3,000 rpm to about 10,000 rpm. In other embodiments, the boundary sedimentation velocity is performed at about 10,000 rpm to about 20,000 rpm. In other embodiments, the boundary sedimentation velocity is performed at about 15,000 rpm to about 20,000 rpm.

[0025] In some embodiments of the above aspects, the boundary sedimentation velocity is performed at about 4°C to about 20 °C. In some embodiments, the boundary sedimentation velocity is performed at about 4°C.

[0026] In some embodiments of the above aspects, the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle. In some embodiments, the recombinant viral particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the recombinant viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In other embodiments, the recombinant viral particle is a recombinant adenoviral particle. In some embodiments, the recombinant adenoviral particle comprises an capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine

Ad, or porcine Ad type 3. In some embodiments, the recombinant adenoviral particle comprises a variant of an adenovirus serotype 2 capsid or a variant of an adenoviral serotype 5 capsid. In other embodiments, the recombinant viral particle is a recombinant lentiviral particle. In some embodiments, the recombinant lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114 or variants therein. In other embodiments, the recombinant viral particle is a rHSV particle. In some embodiments, the HSV particle is an HSV-1 particle or an HSV-2 particle.

[0027] In some aspects, the invention provides methods for evaluating a process for the production of recombinant viral particles comprising the method of any one of claims 1 to 67, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles. In some embodiments, the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle. In some embodiments, the rAAV particles are produced from a producer cell line. In other embodiments, the rAAV particles are produced by triple transfection of i) nucleic acid encoding AAV rep and cap, ii) rAAV vector sequences, and iii) nucleic acid encoding adenovirus helper functions. In other embodiments, the recombinant viral particles are produced by an AAV/HSV hybrid. In other embodiments, the recombinant viral particles are produced from a baculovirus cell. In some embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell. In some embodiments, the recombinant viral particles are produced by introduction of one or more nucleic acids encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell, wherein the one or more nucleic acids are introduced to the cell using a recombinant helper virus. In some embodiments, the recombinant helper virus is an adenovirus or a herpes simplex virus. In some embodiments, the recombinant viral particles comprise a self-complementary AAV (scAAV) genome. In some embodiments, the method is

used to detect the presence of recombinant viral particles comprising the monomeric form of a scAAV genome or the dimeric form of a scAAV genome.

[0028] In some embodiments of the above aspect, the recombinant viral particles are produced by transient transfection of nucleic acid encoding adenovirus vector sequences and adenovirus replication and packaging sequences to a suitable host cell. In other embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding lentivirus vector sequences and/or lentivirus replication and packaging sequences to a suitable host cell. In other embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding HSV vector sequences and/or HSV replication and packaging sequences to a suitable host cell.

[0029] In some aspects the invention provides methods for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release recombinant viral particles; c) isolating the recombinant viral particles produced by the host cell; and d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the above methods. In some aspects the invention provides methods for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release recombinant viral particles; c) isolating the recombinant viral particles produced by the host cell; and d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the above methods. In some embodiments, the p5 promoter is located 3' to the rep and/or cap coding region.

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In some embodiments, the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

[0030] In some embodiments, of any of the preceding embodiments, the recombinant viral particles have been purified using one or more purification steps.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] **FIGS. 1A and 1B** show that analytical ultracentrifugation (AUC) can be used to characterize recombinant viral vector particles. (**FIG. 1A**) A representative scanning profile of boundary sedimentation velocity depicting the absorbance (260 nm) versus the radius (cm) of an AAV2 mixture over a time interval (T) of 1.2 hours. The AAV2 mixture contained empty capsids ("Empty Cap") and full genome capsids ("Intact Vector"). (**FIG. 1B**) A plot of concentration in units of detection, C(S), versus sedimentation coefficient (Svedberg units, S) showing that AUC can be used to measure the concentration of empty capsids and full genome capsids from an 80%/20% mixture. Each peak is labeled with the particle species and its corresponding sedimentation coefficient (S) and relative abundance (%).

[0032] **FIGS. 2A and 2B** show the AUC profiles of pure populations of empty AAV2 capsids (**FIG. 2A**) and genome-containing AAV2-transgene 1 capsids (**FIG. 2B**). Each peak is labeled with the capsid species and its sedimentation coefficient (S).

[0033] **FIGS. 3A and 3B** show a comparison between interference and absorbance detection methods by AUC. (**FIG. 3A**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a 1:1 mixture of empty and genome-containing capsids generated using interference optical detection. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**FIG. 3B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a 1:1 mixture of empty and genome-containing capsids generated using absorbance optical detection (260 nm). The sedimentation coefficient and relative abundance (%) for each species are labeled.

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[0034] **FIG. 4** illustrates the triple transfection method for AAV vector production. The three vectors, containing the gene of interest ("pVector"), AAV Rep and Cap genes ("pHLP"), and adenoviral components ("pIAdeno") are labeled. Note that both genome-containing (labeled with the "ITR-Transgene-ITR" graphic) and empty capsids (blank) are produced.

[0035] **FIG. 5** illustrates the producer cell line method for AAV vector production. As labeled, the HeLa S3 cell line contains integrated Rep, Cap, and Puromycin resistance genes, along with an ITR-flanked transgene of interest. This cell line is infected with adenovirus ("*Ad5*") to stimulate recombinant viral production. Note that both genome-containing (labeled "recombinant viral Vector") and empty capsids are produced, in addition to adenovirus particles.

[0036] **FIGS. 6A, 6B and 6C** shows that vector production by the producer cell line and triple transfection methods yields different vector preparations, as revealed by AUC analysis. (**FIG. 6A**) A schematic of the AAV2-transgene 2 vector and its 3.4 kb genome. (**FIG. 6B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation produced by the producer cell line method. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**FIG. 6C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation produced by the triple transfection method. The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0037] **FIGS. 7A, 7B and 7C** show that the AUC method may be used to monitor the quality and efficacy of vector purification. (**FIG. 7A**) A plot showing the purification of full-genome AAV2-transgene 1 capsids from empty capsids using anion exchange chromatography. Peak fractions corresponding to each species are labeled. (**FIG. 7B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation after elution from the anion exchange column. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**FIG. 7C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation before chromatography. The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0038] **FIG. 8** shows the linear relationship between sedimentation coefficient and vector genome size. A standard curve plotting sedimentation coefficient (S) versus genome size is depicted, along with a line of best fit, its formula, and its associated R^2 value.

[0039] **FIGS. 9A, 9B and 9C** show that assessment of capsid genome size using AUC data correlates with assessment of genome size by Southern blot. (**FIG. 9A**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV9 EGFP vector preparation. Single stranded monomeric (82S) and double stranded dimeric (101S) species are labeled with the corresponding sedimentation coefficients and relative abundance values (%). A schematic of the vector is also provided. (**FIG. 9B**) Alkaline Southern blot analysis of the DNA from scAAV9 EGFP (lane 1) and single stranded AAV9 EGFP (lane 2) vector capsids. Corresponding bands are labeled as described in the blot legend. 4.2 and 2.4 kb size standards are provided as labeled. (**FIG. 9C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a single stranded AAV9 EGFP vector preparation. 82S and 99S (full genome) peaks are labeled with the corresponding sedimentation coefficient and relative abundance values (%).

[0040] **FIGS. 10A, 10B and 10C** show that the Rep/Cap promoter position affects genome packaging in recombinant viral vectors produced by the triple transfection method. (**FIG. 10A**) A schematic of the self-complementary scAAV2 EGFP vector, with estimated sedimentation coefficients for the dimeric and monomeric genome species. (**FIG. 10B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV2 EGFP vector preparation produced using a "wild-type" helper plasmid with the endogenous p5 promoter driving Rep 78/68 expression ("WT Rep"). Peaks for single stranded monomeric (80S) and double stranded dimeric (100S) species are labeled with the corresponding relative abundance values (%). (**FIG. 10C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV2 EGFP vector preparation produced using a "wild-type" helper plasmid with the p5 promoter driving Rep 78/68 expression moved downstream of the cap2 sequence ("pHLP Rep"). Peaks for single

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stranded monomeric (82S) and double stranded dimeric (100S) species are labeled with the corresponding relative abundance values (%).

[0041] **FIGS. 11A, 11B, 11C and 11D** show that the Rep/Cap promoter position affects genome packaging in two additional AAV vectors. (**FIGS. 11A and 11B**) Plots of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yield the distribution of sedimentation coefficients for the single stranded AAV5 Factor IX vector (AAV5 hFIX16) containing a cap5 sequence produced with a helper plasmid having an endogenous p5 promoter ("WT Rep," **FIG. 11B**) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," **FIG. 11A**). (**FIGS. 11C-11D**) Plots of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yield the distribution of sedimentation coefficients for the single stranded AAV5hSMN vector (AAV5SMN) containing a cap5 sequence produced with a helper plasmid having an endogenous p5 promoter ("WT Rep," **FIG. 11D**) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," **FIG. 11C**).

[0042] **FIGS. 12A and 12B** reveal that Southern blot analysis correlates with AUC analysis but misses some fragmented genomes detectable by AUC. (**FIG. 12A**) Southern blot analysis of vector DNA from AAV5SMN preparations made with the pHLP helper plasmid (lane 2) or the WT Rep plasmid (lane 1). 4.6 and 2.4 kb size standards are provided as labeled. (**FIG. 12B**) Southern blot analysis of vector DNA from AAV5FIX preparations made with the pHLP helper plasmid (lane 1) or the WT Rep plasmid (lane 2). 4.3, 3.0, and 1.9 kb size standards are provided as labeled.

[0043] **FIG. 13** provides a map of the AAV5 Factor IX vector indicating the positions of the hFIX transgene, ITR, Rep origin, and *AmpR* marker gene, among other features. Note that the *AmpR* marker is upstream of the ITR, enhancer, and promoter region.

[0044] **FIGS. 14A and 14B** show that WT Rep vector genomes, unlike pHLP Rep vector genomes, package sequences upstream of the 5' ITR in the AAV5 Factor IX vector. (**FIG. 14A**) Southern blot analysis using an hFIX transgene-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes. (**FIG. 14B**) Southern blot analysis using a Rep ori/*AmpR*-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes.

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[0045] **FIGS. 15A and 15B** show the fragmentation of oversized AAV vector genomes, as demonstrated by AUC analysis. (**FIG. 15A**) Plot of concentration, C(S), versus sedimentation coefficient (S) generated by AUC for an AAV vector with an oversized genome. This genome contains a full-length chicken β-actin (CBA) promoter driving expression of β-phosphodiesterase (ssAAV2/5CBA-βPDE). Peaks for detected species are labeled by observed sedimentation coefficient (S) and relative abundance values (%). (**FIG. 15B**) Plot of concentration, C(S), versus sedimentation coefficient (S) generated by AUC for an AAV vector with a truncated genome. This genome contains a CBA promoter with a reduced-size intron driving expression of β-phosphodiesterase (AAV5 minCBAPDE6B). Peaks for detected species are labeled by observed sedimentation coefficient (S) and relative abundance values (%).

[0046] **FIG. 16** shows the AUC profiles of pure populations of adenovirus capsids. The sedimentation coefficient (S) and interference values are given for each peak.

DETAILED DESCRIPTION

[0047] The present invention provides methods of characterizing preparations of viral particles using analytical ultracentrifugation. By subjecting preparations to analytical ultracentrifugation (AUC) under boundary sedimentation velocity conditions, the sedimentation of viral particles can be monitored at time intervals (*e.g.*, one or more times). The differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S) is then plotted and the area under each peak in the C(s) distribution is integrated to determine the relative concentration of each peak. Each peak represents a species of viral particle reflective of its molecular weight. The species that can be detected by these methods include, but are not limited to, recombinant adeno-associated viral (rAAV) particles, recombinant adenoviral (rAd) particles, recombinant lentiviral particles, and recombinant herpes simplex viral (rHSV) particles. To use rAAV particles as an illustrative example, these methods allow the detection of rAAV species including rAAV capsid particles comprising intact rAAV genomes (*e.g.*, full capsids), empty viral capsids wherein no rAAV genomes have been encapsidated into viral capsids, and rAAV particle variants in which variant rAAV genomes are encapsidated in viral capsids (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates, and the like). These methods can be applied to preparations of viral particles regardless of nucleotide sequence of the viral genome or, in the case of recombinant viral

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particles, the serotype of the recombinant viral capsid. These methods can be applied to rAAV, rAd, recombinant lentivirus and rHSV viral particles.

[0048] In some aspects, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles by subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times). By plotting the differential sedimentation coefficient distribution value C(S) versus the sedimentation coefficient in Svedberg units (S), species of recombinant viral particles in the preparation can be identified by presence of peaks on the plot corresponding to an S value. The genome size of a particular species of recombinant viral particles can be calculated, for example, by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of different known size. The vector genomes that can be assessed by these methods include, but are not limited to, recombinant viral capsid particles comprising intact recombinant viral genomes (*e.g.*, full capsids), empty viral capsids wherein no recombinant viral genomes have been encapsidated into viral capsids, and recombinant viral particle variants in which variant recombinant viral genomes (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates and the like) are encapsidated in viral capsids. In some embodiments, the viral particles are rAAV, rAd, recombinant lentivirus or rHSV viral particles.

[0049] In some embodiments the invention provides methods of determining the heterogeneity of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) in a preparation of recombinant viral particles by AUC under boundary sedimentation velocity conditions wherein the presence of peaks in a plot of C(S) v. S, in addition to the peak representing capsids comprising an intact viral genome, indicates heterogeneity of recombinant viral particles in the preparation. In some embodiments, the relative amounts of each recombinant viral species in the preparation are calculated by integrating the area for each peak in the plot.

[0050] In some embodiments of the invention, AUC is used to determine the presence of empty capsids and/or recombinant viral particle variants in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles), wherein the presence of peak that corresponds to the S value of empty capsid particles and/or recombinant viral particle variants in a

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plot of C(S) vs. S indicates the presence of empty capsid particles and/or recombinant viral particle variants. In some embodiments, the relative amount of empty capsids and/or recombinant viral particle variants in a preparation of recombinant viral particles is determined by integrating the area under each peak in a plot of C(S) versus S and comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles and/or recombinant viral particle variants to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value corresponding to empty capsid particles and/or recombinant viral particle variants is compared to the total amount of all recombinant viral particles in the preparation by integrating and summing and the area under all the peaks of the plot.

[0051] In some embodiments, the invention provides methods of monitoring the removal of empty capsids and/or recombinant viral particle variants during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) by using AUC. Samples of the recombinant viral particles from the preparation following one or more steps in the purification process are analyzed for the relative amount of empty capsids and/or recombinant viral particle variants wherein a decrease in the relative amount of empty capsids and/or recombinant viral particle variants to full capsid particles indicates removal of empty capsids and/or recombinant viral particle variants from the preparation of recombinant viral particles.

[0052] In some embodiments, the invention provides methods of evaluating processes for the production of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) by AUC. The preparation of recombinant viral particles is analyzed for the presence of intact full viral capsid particles, empty particles and/or recombinant viral particle variants. An increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral particle variants (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates, and the like) compared to a reference preparation of recombinant viral particles (*e.g.*, a standard recombinant viral preparation process) indicates an improvement in the production of recombinant viral particles.

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I. General Techniques

[0053] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, *et al.* eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Ausubel *et al.*, eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway *et al.*, 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita *et al.*, eds., J.B. Lippincott Company, 2011).

II. Definitions

[0054] A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

[0055] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term

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includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate- phosphodiester oligomer. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0056] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0057] A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs).

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[0058] A "recombinant AAV vector (recombinant adeno-associated viral vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one AAV inverted terminal repeat sequences (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (*i.e.* AAV Rep and Cap proteins). When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an AAV particle. A recombinant viral vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (recombinant viral particle)".

[0059] An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

[0060] A "recombinant adenoviral vector" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of adenovirus origin) that are flanked by at least one adenovirus inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that is expressing essential adenovirus genes deleted from the recombinant viral genome (*e.g.*, E1 genes, E2 genes, E4 genes, etc.). When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of adenovirus packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an adenovirus

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particle. A recombinant viral vector can be packaged into an adenovirus virus capsid to generate a "recombinant adenoviral particle."

[0061] A "recombinant lentivirus vector" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentivirus origin) that are flanked by at least one lentivirus terminal repeat sequences (LTRs). In some embodiments, the recombinant nucleic acid is flanked by two lentiviral terminal repeat sequences (LTRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. A recombinant lentiviral vector can be packaged into a lentivirus capsid to generate a "recombinant lentiviral particle."

[0062] A "recombinant herpes simplex vector (recombinant HSV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of HSV origin) that are flanked by HSV terminal repeat sequences. Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of HSV packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an HSV particle. A recombinant viral vector can be packaged into an HSV capsid to generate a "recombinant herpes simplex viral particle."

[0063] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (*e.g.*, a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0064] The term "transgene" refers to a polynucleotide that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

[0065] The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant viral DNA genome or RNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark *et al.* (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk *et al.* (2002) *Mol. Ther.*, 6:272-278.

[0066] The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant viral vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example with AAV, in McLaughlin *et al.* (1988) *J. Virol.*, 62:1963-1973.

[0067] The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant viral vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example regarding AAV, in Xiao *et al.* (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher *et al.* (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0068] An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0069] An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several

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shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0070] A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins.

[0071] "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0072] A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A helper virus provides "helper functions" which allow for the replication of AAV. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Examples of adenovirus helper functions for the replication of AAV include E1A functions, E1B functions, E2A functions, VA functions and E4orf6 functions. Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

[0073] A preparation of rAAV is said to be "substantially free" of helper virus if the ratio of infectious AAV particles to infectious helper virus particles is at least about 10^2:1; at least about 10^4:1, at least about 10^6:1; or at least about 10^8:1 or more. In some embodiments, preparations are also free of equivalent amounts of helper virus proteins (*i.e.*, proteins as would be present as a result of such a level of helper virus if the helper virus particle impurities noted above were present in disrupted form). Viral and/or cellular protein contamination can generally be observed

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as the presence of Coomassie staining bands on SDS gels (*e.g.*, the appearance of bands other than those corresponding to the AAV capsid proteins VPl, VP2 and VP3).

[0074] As used herein, "differential coefficient distribution value" or "C(S)" is a variant of the distribution of Lamm equation solutions to describe distributions of sedimenting particles; for example during ultracentrifugation.

[0075] As used herein, "Svedberg units" refers to a unit for sedimentation rate. The sedimentation rate for a particle of a given size and shape measures how fast the particle sediments. One Svedberg unit is equivalent to 10^{-13} seconds. For example, Svedberg units are often used to reflect the rate at which a molecule travels under the centrifugal force of a centrifuge.

[0076] As used herein, "sedimentation velocity conditions" or "boundary sedimentation velocity conditions" may refer to any experimental conditions under which a sample solution is subjected to sedimentation velocity analysis. Sedimentation velocity allows the study of particles over a wide range of pH and ionic strength conditions and at temperatures 4 to 40 °C. The rate at which the sedimentation boundary moves is a measure of the sedimentation coefficient of the sedimenting species. The sedimentation coefficient depends on the molecular weight (larger particles sediment faster) and also on molecular shape. The minimum width of the sedimentation boundary is related to the diffusion coefficient of the molecule; the presence of multiple species with similar sedimentation coefficients will cause the boundary to be broader than expected on the basis of diffusion alone. Sedimentation velocity conditions may include without limitation any conditions related to the rotor speed, distance between sample and rotor center, temperature, solvent, sample, buffer, ultracentrifugation time, time interval for detection, sector and optical window characteristics, AUC instrumentation (including ultracentrifuge and detection apparatus), equilibrium dialysis of reference solvent, and data analysis algorithms.

[0077] As used herein, the term "analytical density gradient sedimentation equilibrium" relates to methods for measuring the buoyant density of a particle, or using differences in buoyant density to separate different species of particles. These methods may use, for example, AUC sedimentation equilibrium techniques. In these methods, a particle solution (*e.g.*, without limitation, a solution of a polypeptide, polynucleotide, or viral capsids) may be subjected to

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ultracentrifugation in a gradient solvate, such as a cesium chloride or cesium sulfate gradient, until equilibrium with the solvate is attained. At equilibrium, the particle solution will concentrate, or band, at the position in the gradient where the density of the particle is equal to that of the solvate. The position of bands may be used to calculate particle density, or a band may be extracted to isolate a single species of particle.

[0078] As used herein, the "SEDFIT algorithm" is an algorithm that allows one to analyze hydrodynamic data such as sedimentation velocity (Schuck (2000) *Biophys. J.*, 78:1606-19). In the SEDFIT algorithm, a grid of sedimentation coefficients across an expected range is created. Sedimentation boundaries are simulated using solutions to the Lamm equation for each sedimentation coefficient, assuming constant particle shape and solvent frictional ratio.

[0079] As used herein, the term "F statistic" or "F ratio" refers to the confidence level. This parameter controls the amount of regularization used. It has a different meaning for different ranges: From 0 to 0.5, no regularization is used. Values from 0.5 to 0.999 correspond to probabilities P (confidence levels). From these P-values, the desired chi-square increase allowed for the parsimony constraint of the regularization is calculated with F-statistics. A value of 0.51 will cause very little regularization; values of 0.68 to 0.90 would correspond to commonly used confidence levels (usually, with 50 scans or more the chi-square increase corresponding to a probability of 0.7 is of the order of 0.1%), while values close to 0.99 would cause very high regularization. The relationship of these values with probabilities can be examined using the F-statistics calculator. If numbers > 1 are entered, they are taken directly as chi-square ratios (as there are no probabilities > 1). For example, a value of 1.1 will result in regularization with 10% chi-square increase.

[0080] To "reduce" is to decrease, reduce or arrest an activity, function, and/or amount as compared to a reference. In certain embodiments, by "reduce" is meant the ability to cause an overall decrease of 20% or greater. In another embodiment, by "reduce" is meant the ability to cause an overall decrease of 50% or greater. In yet another embodiment, by "reduce" is meant the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater.

[0081] A "reference" as used herein, refers to any sample, standard, or level that is used for comparison purposes. For example, when measuring absorbance or refraction of AAV in an

aqueous solution, the absorbance or refraction of the solution is compared to the absorbance or refraction of the aqueous solution without AAV (*i.e.* a reference solution). In other examples, a reference may refer to a standard procedure known in the art. For example, when analyzing a procedure for improved quality of AAV production (*e.g.*, homogeneity), the AAV produced by the candidate procedure is compared to procedures known in the art (*i.e.* reference procedures).

[0082] An "isolated" molecule (*e.g.*, nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment. Thus, for example, isolated rAAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like, as defined below.

[0083] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X."

[0084] As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise. For example, the phrase "a rAAV particle" includes one or more rAAV particles.

[0085] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Analytical ultracentrifugation

[0086] Analytical ultracentrifugation is a means to evaluate the molecular weight and the hydrodynamic and thermodynamic properties of a protein or other macromolecule. Heterogeneity of a protein or macromolecule by sedimentation velocity over a range of conditions including concentration, temperature, ionic strength, and pH. For example, a protein may be analyzed in a

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clinically relevant formulation. Use of analytical ultracentrifugation to characterize adenovirus preparations is provided by Berkowitz, SA & Philo JS, (2007) *Anal. Biochem.*, 362:16-37.

[0087] In certain aspects, the present invention provides methods of characterizing preparations of viral particles using analytical ultracentrifugation (AUC). For example, in some embodiments, the invention provides methods to assess vector genome integrity of recombinant adeno-associated viral (rAAV) particles in preparations of rAAV particles using AUC to distinguish viral particles with full, intact genomes, empty viral capsids and viral particles with variant (*e.g.*, truncated, aggregates, impurities and the like) viral genomes. In others embodiments, these methods may be applied in a similar way to analyze adenovirus, lentivirus, and herpes simplex virus (HSV) particles. AUC analysis refers to quantitative methods for characterizing the biophysical properties of particles (*e.g.*, polypeptides, polynucleotides, and viral capsids) by measuring their migration through a solvent in a centrifugal field. AUC analysis has been well characterized over many decades and is highly versatile. Because AUC analysis relies upon first-principle hydrodynamic and thermodynamic information, AUC may be applied to determine the biophysical properties of many types of particles across a wide range of particle concentrations and sizes. AUC analysis typically encompasses two basic types of experiment: sedimentation velocity and sedimentation equilibrium. Sedimentation equilibrium analysis yields thermodynamic properties of particles that may be used to measure characteristics such as stoichiometry and association constants. Sedimentation velocity yields hydrodynamic properties of particles that may be used to measure characteristics such as size, shape, and concentration. A feature of AUC analysis of viral preparations is that the same assay conditions may be used to analyze different preparations of viral particles regardless of nucleotide sequence of the viral genome or serotype of the capsid.

[0088] Certain aspects of the present disclosure relate to the use of sedimentation velocity analysis to characterize viral capsid properties. In some embodiments, sedimentation velocity analysis uses an ultracentrifuge velocity cell with two sectors in dialysis equilibrium (one for an experimental sample and one for a solvent-only reference sample), each containing two optical windows that allow light to pass through the compartment. Ultracentrifugation applies an angular velocity to the cell and leads to rapid sedimentation of the solute particles towards the bottom of the sector. As sedimentation occurs, solute is depleted near the meniscus at the top of the cell, creating a sedimenting boundary between the depleted region and the sedimenting solute. The

rate of movement or migration of the sedimenting boundary is measured by taking measurements that compare the properties of the sample and reference sectors at specific time intervals (for sedimentation velocity, these intervals are typically on the order of minutes). If multiple species of solute are present, this may lead to the formation of multiple sedimenting boundaries, each corresponding to a resolvable species.

[0089] Several methods for optically detecting a sedimenting boundary and measuring its rate of movement or migration are known in the art (for reference, see Cole *et al.* (2008) *Methods Cell Biol.*, 84:143-79). In some embodiments, the reference and sample sectors may be assayed using absorbance detection. In this detection method, the absorbance at a particular wavelength may be measured for the sample and reference sectors at different radial positions within each sector. Alternatively, the time course of absorbance at a single radial position may be measured. Beer's Law provides a mathematical relationship between absorbance and a solute's extinction coefficient.

[0090] In some embodiments, the reference and sample sectors may be assayed using interference detection (*e.g.*, Rayleigh interference detection). In the Rayleigh interference detection method, the interference optical system contains two parallel slits. A single, coherent beam of light is split such that it passes through both windows, and then the two beams are re-merged. When these two light waves are merged, they form an interference pattern of alternating light and dark fringes. If the sample and reference samples were to have an identical refractive index, the resulting interference fringes would be perfectly straight. Increasing the concentration of solute increases the solution's refractive index, thereby retarding the sample light beam and causing a vertical fringe shift. By measuring this fringe shift, one may measure the concentration of solute in the sample. Unlike absorbance detection, which measures absolute values for the sample and reference, interference detection measures a relative difference between the sample and reference. However, interference detection yields integrated peaks that are directly proportional to concentration, and it may be used for types of samples that do not absorb significantly. For a reference on using Rayleigh interference optics with AUC, see Furst (1997) *Eur. Biophys. J.* 35:307-10.

[0091] Measurement of the rate at which the sedimentation boundary moves may be used to derive many physical properties of solute particles. The rate of the boundary movement

determines the sedimentation coefficient, which is based on the mass and shape (frictional coefficient) of the particle. The sedimentation coefficient of a particle, *s*, refers to the ratio of its velocity to the acceleration applied to it by a centrifugal field. Sedimentation coefficients are expressed in Svedberg units, S (one Svedberg unit is equivalent to 10^{-13} seconds). The sedimentation coefficient of a particle or solution of particles depends upon its properties, for example molecular weight (corrected for buoyancy), and the properties of the solvent.

[0092] The change in the concentration boundary of a solute over time during ultracentrifugation may be determined using the Lamm equation (Schuck (2000) *Biophys. J.*, 78:1606-19). Briefly, the Lamm equation calculates the change in the concentration boundary of a solute over time in response to the competing forces of sedimentation (which concentrates the solute) and diffusion (which disperses the solute), taking into account the sector-shaped cell and the centrifugal field generated by the rotor. The Lamm equation may be expressed as:

Equation 1: $\partial c/\partial t = D\,[(\partial^2 c/\partial r^2) + 1/r(\partial c/\partial r)] - s\omega^2\,[r(\partial c/\partial r) + 2c]$

where c is the solute concentration, D represents the solute diffusion constant, s represents the sedimentation coefficient, ω represents the angular velocity of the rotor, r is the radius, and t is time.

[0093] By fitting raw AUC data to solutions of the Lamm equation, it is possible to determine solute characteristics such as the sedimentation coefficient and the change in concentration distribution. For example, experimentally determined values for the rate of change of a sedimenting boundary may be modeled using the Lamm equation to derive the sedimentation coefficient, molecular mass, or concentration of the solute forming the boundary. Several programs known in the art, such as SEDFIT (Schuck (2000) *Biophys. J.*, 78:1606-19), may be used to model the Lamm equation to AUC data. These programs are also able to apply the Lamm equation to solutions containing multiple solutes or multiple sedimenting boundaries.

[0094] One example of a suitable program for the determination of solute characteristics is the SEDFIT algorithm. In some embodiments, the SEDFIT algorithm may be used to calculate a differential coefficient distribution value, or C(S), using AUC data from a solution containing a mixture of particle species (for reference, see Schuck (2000) *Biophys. J.*, 78:1606-19). In the SEDFIT algorithm, a grid of sedimentation coefficients across an expected range is created.

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Sedimentation boundaries are simulated using solutions to the Lamm equation for each sedimentation coefficient, assuming constant particle shape and solvent frictional ratio. Actual AUC data are then fit to these Lamm solutions to derive the differential coefficient distribution value, or C(S). Many other programs useful for analyzing AUC data may be found in Cole and Hansen (1999) *J. Biomol. Tech.* 10:163-76.

[0095] In some embodiments of the invention, recombinant viral particles are highly purified, suitably buffered, and concentrated. In some embodiments, the viral particles are concentrated to at least about any of 1 x 10^7 vg/mL, 2 x 10^7 vg/mL, 3 x 10^7 vg/mL, 4 x 10^7 vg/mL, 5 x 10^7 vg/mL, 6 x 10^7 vg/mL, 7 x 10^7 vg/mL, 8 x 10^7 vg/mL, 9 x 10^7 vg/mL, 1 x 10^8 vg/mL, 2 x 10^8 vg/mL, 3 x 10^8 vg/mL, 4 x 10^8 vg/mL, 5 x 10^8 vg/mL, 6 x 10^8 vg/mL, 7 x 10^8 vg/mL, 8 x 10^8 vg/mL, 9 x 10^8 vg/mL, 1 x 10^9 vg/mL, 2 x 10^9 vg/mL, 3 x 10^9 vg/mL, 4 x 10^9 vg/mL, 5 x 10^9 vg/mL, 6 x 10^9 vg/mL, 7 x 10^9 vg/mL, 8 x 10^9 vg/mL, 9 x 10^9 vg/mL, 1 x 10^{10} vg/mL, 2 x 10^{10} vg/mL, 3 x 10^{10} vg/mL, 4 x 10^{10} vg/mL, 5 x 10^{10} vg/mL, 6 x 10^{10} vg/mL, 7 x 10^{10} vg/mL, 8 x 10^{10} vg/mL, 9 x 10^{10} vg/mL, 1 x 10^{11} vg/mL, 2 x 10^{11} vg/mL, 3 x 10^{11} vg/mL, 4 x 10^{11} vg/mL, 5 x 10^{11} vg/mL, 6 x 10^{11} vg/mL, 7 x 10^{11} vg/mL, 8 x 10^{11} vg/mL, 9 x 10^{11} vg/mL, 1 x 10^{12} vg/mL, 2 x 10^{12} vg/mL, 3 x 10^{12} vg/mL, 4 x 10^{12} vg/mL, 5 x 10^{12} vg/mL, 6 x 10^{12} vg/mL, 7 x 10^{12} vg/mL, 8 x 10^{12} vg/mL, 9 x 10^{12} vg/mL, 1 x 10^{13} vg/mL, 2 x 10^{13} vg/mL, 3 x 10^{13} vg/mL, 4 x 10^{13} vg/mL, 5 x 10^{13} vg/mL, 6 x 10^{13} vg/mL, 7 x 10^{13} vg/mL, 8 x 10^{13} vg/mL, 9 x 10^{13} vg/mL. In some embodiments, the viral particles are concentrated to of about 1 x 10^7 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{11} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{12} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^{11} vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^9 vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^9 vg/mL, or about 1 x 10^7 vg/mL to about 1 x 10^8 vg/mL.

[0096] In some embodiments, viral particles are generated in a suitable host cells and purified. In some embodiments, the viral particles are purified by affinity chromatography. Methods to

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purify viral particles (*e.g.*, AAV particles, adenovirus particles, lentivirus particles, HSV particles) are known in the art. For example, by use of an antibody of a viral capsid protein or binding ligand of a viral capsid protein immobilized on a chromatography media. Examples of viral capsid affinity chromatographies include but are not limited to AVB affinity chromatography for AAV (GE Healthcare), metal affinity chromatography for adenovirus and HSV, and heparin affinity chromatography for AAV and lentivirus, and the like. Methods to purify adenovirus particles are found, for example, in Bo, H *et al.*, (2014) *Eur. J. Pharm. Sci.* 67C:119-125. Methods to purify lentivirus particles are found, for example, in Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for example, in Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79.

[0097] In some embodiments, the recombinant viral particles are formulated in a pharmaceutical composition. In related embodiments, the pharmaceutical composition contains a buffer having physiological pH and/or physiological osmolality. A nonlimiting example of a pharmaceutical formulation is phosphate buffered saline (PBS) and in some embodiments, the PBS can be at physiological osmolality (*e.g.*, about pH 7.2 and about 300 mOsm/L). In some embodiments, sample adjustments are made to target concentration by optical density measurement at 260nm from 0.1 to 1.0. In some examples, this concentration results in reproducible and consistent AUC data. In some examples, concentration of viral particles is adjusted either by direct dilution with PBS or further concentration; for example, by using a centrifugal filter device.

[0098] In some embodiments of the invention, sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis is performed using an analytical ultracentrifuge that is capable of characterizing a sample in its native state under biologically relevant solution conditions (*e.g.*, ProteomeLab™ XL-I (Beckman Coulter)). When using the ProteomeLab™ XL-I, sample is loaded into the sample sector of a two sector velocity cell, a vehicle control (*e.g.*, PBS without recombinant viral) is loaded into the corresponding reference sector. The sample is placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of about 20 °C and full vacuum are maintained for about one hour. In an exemplary embodiment, sedimentation velocity centrifugation is performed at about 20,000 RPM, about 20 °C, and about 0.003 cm radial step setting, with no delay and with no replicates. As noted below, different parameters may be used for centrifugation. In some embodiments, absorbance (260 nm) and/or

interference optics (*e.g.*, Rayleigh interference optics) are used to simultaneously record radial concentration as a function of time until the smallest sedimenting component clears the optical window. In some embodiments, the radial concentration is recorded until the sedimenting species with the lowest density clears the sector. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge. A sector may be a portion of an ultracentrifuge; for example an ultracentrifuge velocity cell. In some embodiments, a sector may be a portion of an ultracentrifuge where samples are detected. In some embodiments, the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell. In some embodiments, is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window. In some embodiments, the radial concentration is recorded for at least about any of 0.5 hours, 0.75 hours, 1.0 hours, 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, or 5.0 hours. In some embodiments, the radial concentration is recorded for between any of about 0.5 hours to about 0.75 hours, about 0.75 hours to about 1.0 hours, about 1.0 hours to about 1.5 hours, about 1.5 hours to about 2.0 hours, about 2 hours to about 3 hours, about 3 hours to about 4 hours, about 4 hours to about 5 hours. In some embodiments, the radial concentration is recorded for about 1.2 hours. Optimizing runs conditions may include, for example, continuing the run until all of the sedimenting species are fully sedimented to the bottom of the sector, with the temperature held constant at 20 °C and a speed between 18,000 rpm and 20,000 rpm. As noted below, other temperatures and speeds may be used.

[0099] The percent full capsid is determined by analyzing a multiple of scans (*e.g.*, 75) from each detection method using the SEDFIT continuous size C(S) distribution model. Second (2nd) derivative regularization is applied to the fitting. In some embodiments, the confidence level of F statistic is about 0.68. In some embodiments, the confidence level of F statistic is more than about any of 0.68, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95 or 0.99. In some embodiments, the confidence level of F statistic is about 0.68 to about 0.90. In some embodiments, the confidence level of F statistic is about 0.68 to about 0.99. In some embodiments, the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software. In some embodiments, the resolution is about any of 200S, 300S,

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400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the resolution is between any of about 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, the resolution is about 200S. In some embodiments, the Smax is about any of 100S, 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the Smax is between any of about 100S to about 1000S, 100S to about 900S, 100S to about 800S, 100S to about 700S, 100S to about 600S, 100S to about 500S, 100S to about 400S, 100S to about 300S, 100S to about 200S, 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, Smax is about 200S to about 5000S. In some embodiments, wherein Smax is about 200S. In some embodiments, the frictional ratio is left to float to a value determined by centrifugation software. In some embodiments, the frictional ratio is about 1.0. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied. In some embodiments, the meniscus position is allowed to float, letting the software choose the optimal position. In some embodiments, the frictional ratio is allowed to float, letting the software choose the optimal position. The model fits the data to the Lamm equation, and the resulting size distribution is a "distribution of sedimentation coefficients" that looks like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) are determined for each component in the distribution. In some

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embodiments, multiple AUC runs are independent assays, and each analysis the following attributes are monitored to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\ nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans.

[0100] In some embodiments of the invention, extinction coefficients are used to calculate molar concentration and the actual percent value of the intact vector peak from absorbance data. Molar absorbance extinction coefficients for both empty capsids ($\epsilon_{260}/_{capsid}$=3.72e6) and intact vector ($\epsilon_{260}/_{vector}$=3.00e7) can be calculated based on published formulae (Sommer *et al.* (2003) *Mol Ther.*, 7:122-8). Extinction coefficients are available for empty capsid and intact vector peaks. The C(S) values can be determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.*, 78:1606-19. Molar concentration of both intact vector and empty capsid can be calculated using Beer's Law and the percentage of full capsid are calculated from these values. In some embodiments, values are reported in terms of the percentage of full capsid.

[0101] In some embodiments, it is not possible to determine empirically the extinction coefficient of particular species of recombinant viral particles (*e.g.*, viral particles with fragmented genomes of unknown size and sequence). A relationship between S value and genome size may be established by analyzing recombinant viral vector preps with encapsidated viral genomes of known nucleotide size and a corresponding S value are determined as described herein. The calculated S values can be plotted to generate a standard curve to which recombinant viral species of unknown molecular weight or genome size can be compared to determine the molecular weight of the unknown species.

[0102] In some aspects, the invention provides methods of characterizing a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle. In some embodiments, the species of recombinant viral particle identified by the methods of the invention include, but are

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not limited to: full recombinant viral particles comprising intact recombinant viral genomes, empty recombinant viral capsid particles, and recombinant viral particles comprising variant recombinant viral genomes. In some embodiments the variant genomes are smaller than the intact recombinant viral genome (*e.g.*, truncated genomes). In some embodiments, the variant genomes are larger than the intact recombinant viral genome (*e.g.*, aggregates, recombinants, *etc.*). In some embodiments, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of different known size. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles. In some embodiments, the sedimentation of recombinant viral particles is monitored at one time interval. In some embodiments, the sedimentation of recombinant viral particles is monitored at more than one time interval.

[0103] In some embodiments of the invention, the sedimentation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) is monitored by measuring optical density or absorbance at about 260 nm. Means of measuring absorbance are known in the art. In some embodiments, an ultracentrifuge used for AUC is equipped with means for measuring absorbance. In other embodiments, the sedimentation of recombinant viral particles is monitored by interference. In some embodiments, the sedimentation of recombinant viral particles is monitored by Rayleigh interference. Means of measuring interference are known in the art (Furst (1997) *Eur. Biophys. J.* 35:307-10). In some embodiments, an ultracentrifuge used for AUC is equipped with means for measuring interference. In some embodiments, the sedimentation of recombinant viral particles is monitored by both absorbance and interference. In some embodiments, the absorbance and/or interference are measured using a reference standard. In some embodiments,

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the reference standard matches the solution of the recombinant viral preparation with the exception that the recombinant viral is not present. For example, the recombinant viral preparation may comprise recombinant viral in a buffer such as phosphate buffered saline. In this example, the reference standard may be phosphate buffered saline without recombinant viral particles.

[0104] In some embodiments of the invention, the preparation of viral particles is in a pharmaceutical formulation. Such formulations are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580). Such pharmaceutical formulations can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical formulation may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. In some embodiments of the invention the pharmaceutical formulation comprises phosphate buffered saline.

[0105] In some embodiments of the invention, the sedimentation velocity of viral particles during ultracentrifugation is determined by monitoring the sedimentation of viral particles continuously during ultracentrifugation. It is within the purview of the skilled artisan to optimize the parameters of AUC for different types of viral particles. Without wishing to be bound to theory, a range of AUC settings that allows the analysis of both AAV and adenovirus particles should enable the analysis of other viral particles including lentivirus and HSV since the size of HSV and lentiviral particles is between that of AAV and adenovirus particles. In some embodiments, data acquisition for rAAV, rHSV, lentiviral, and/or rAd particles is performed with an AUC speed of between about 3,000 and about 20,000 rpm. In some embodiments, data analysis for rAAV, HSV, lentiviral, and/or adenoviral particles is performed with an S_{min} of about 1S and an S_{max} of about 1000S. In some embodiments, data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with a resolution of about 200S to about 1,000S. In some embodiments, the resolution is about any of 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the resolution is between any of about 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S,

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200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, the resolution is about 200S. data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with an Smax of about any of 100S, 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the Smax is between any of about 100S to about 1000S, 100S to about 900S, 100S to about 800S, 100S to about 700S, 100S to about 600S, 100S to about 500S, 100S to about 400S, 100S to about 300S, 100S to about 200S, 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, Smax is about 200S to about 5000S. In some embodiments, wherein Smax is about 200S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied. In some embodiments, the meniscus position is allowed to float, letting the software choose the optimal position. In some embodiments, the frictional ratio is allowed to float, letting the software choose the optimal position. In some embodiments, data analysis for rAAV and/or adenoviral particles is held constant at 1. In some embodiments, data analysis for rAAV, HSV, lentiviral, and/or adenoviral particles is allowed to float by using the FIT command with a value optimized using non-linear regression.

[0106] With respect to recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles), in some embodiments, the sedimentation velocity of recombinant viral during ultracentrifugation is determined by monitoring (*e.g.*, scanning) the sedimentation of recombinant viral particles once in more than about every 15 seconds, 30 seconds, 45 seconds, 1 minute (60

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seconds), 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes. Scans may be continuously acquired without delay as quickly as the optical systems allow. Interference scans are rapid, and a single scan is complete in ~10-15 seconds, while absorbance scans require ~ 60 seconds. When dual detection is used the speed of scan acquisition for both are determined by the absorbance system. In some embodiments of the invention, more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 scans are used to monitor sedimentation of recombinant viral particles during ultracentrifugation. In some embodiments, a minimum of 30 scans is required for analysis, and scans are collected until the sedimentation process is complete. In some embodiments, the sedimentation process may typically be described by between 40 and 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 55 scans to about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 55 scans to about 60 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 60 scans to about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 60 scans to about 70 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on multiple ultracentrifugations (runs). In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more ultracentrifugation runs. In some embodiments, the sedimentation velocities are used to determine C(S) values using the SEDFIT algorithm. In some embodiments, a second derivative regularization is applied to a fitting level with a confidence level of F statistic of about 0.68. In some embodiments, the following C(S) parameters are held constant: resolution 100S to about 200S, S min is about 1, S max is about 200S to 300S, and frictional ratio is about 1.0 to 1.2S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

[0107] In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at more than about any of 5,000 rpm; 10,000 rpm; 15,000 rpm; 20,000 rpm; 25,000 rpm; 30,000 rpm; 35,000 rpm; 40,000 rpm; 45,000 rpm; or 50,000 rpm. In some embodiments, the

ultracentrifugation is run at between any of about 5,000 rpm and about 50,000 rpm; about 10,000 rpm and about 50,000 rpm; about 15,000 rpm and about 50,000 rpm; about 20,000 rpm and about 50,000 rpm; about 25,000 rpm and about 50,000 rpm; about 30,000 rpm and about 50,000 rpm; about 35,000 rpm and about 50,000 rpm; about 40,000 rpm and about 50,000 rpm; about 45,000 rpm and about 50,000 rpm; about 5,000 rpm and about 45,000 rpm; about 10,000 rpm and about 45,000 rpm; about 15,000 rpm and about 45,000 rpm; about 20,000 rpm and about 45,000 rpm; about 25,000 rpm and about 45,000 rpm; about 30,000 rpm and about 45,000 rpm; about 40,000 rpm and about 45,000 rpm; about 5,000 rpm and about 40,000 rpm; about 10,000 rpm and about 40,000 rpm; about 15,000 rpm and about 40,000 rpm; about 20,000 rpm and about 40,000 rpm; about 25,000 rpm and about 40,000 rpm; about 30,000 rpm and about 40,000 rpm; about 35,000 rpm and about 40,000 rpm; about 5,000 rpm and about 35,000 rpm; about 10,000 rpm and about 35,000 rpm; about 15,000 rpm and about 35,000 rpm; about 20,000 rpm and about 35,000 rpm; about 25,000 rpm and about 35,000 rpm; about 30,000 rpm and about 35,000 rpm; about 5,000 rpm and about 30,000 rpm; about 10,000 rpm and about 30,000 rpm; about 15,000 rpm and about 30,000 rpm; about 20,000 rpm and about 30,000 rpm; about 25,000 rpm and about 30,000 rpm; about 5,000 rpm and about 25,000 rpm; about 10,000 rpm and about 25,000 rpm; about 20,000 rpm and about 25,000 rpm; about 5,000 rpm and about 20,000 rpm; about 10,000 rpm and about 20,000 rpm; about 15,000 rpm and about 20,000 rpm; about 5,000 rpm and about 15,000 rpm; about 10,000 rpm and about 15,000 rpm; or about 5,000 rpm and about 10,000 rpm. In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 20,000 rpm. In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 15,000 rpm to about 20,000 rpm.

[0108] In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) by ultracentrifuging the preparation of recombinant viral particles at about or more than 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, or 30 °C. In some embodiments, the ultracentrifugation is run as between any of about 4 °C and about 30 °C, about 4 °C and about 25 °C, about 4 °C and about 20 °C, about 4 °C and about 15 °C, about 4 °C and about 10 °C, about

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10 °C and about 30 °C, about 10 °C and about 25 °C, about 10 °C and about 20 °C, about 10 °C and about 15 °C, about 15 °C and about 30 °C, about 15 °C and about 25 °C, about 15 °C and about 20 °C, about 20 °C and about 30 °C, or about 20 °C and about 25 °C. In some embodiments, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 20 °C. In some embodiments, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 15 °C to about 20 °C.

[0109] As disclosed herein, numerous types of recombinant viral particles may be analyzed by the methods of the present disclosure (*e.g.*, AAV, adenoviral, lentiviral, and/or HSV particles). Suitable ultracentrifugation conditions, analysis algorithms, and other parameters may be determined empirically through methods known in the art. Exemplary parameters for AAV, adenoviral, lentiviral, and HSV particles, along with guidance for the selection of specific parameter options, are provided without limitation in Table 1 below.

Table 1. Exemplary parameters for AAV, adenoviral, lentiviral, and HSV particles.

	AAV	Ad	Lentivirus	HSV
Exemplary buffers	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L
Exemplary algorithms for determining C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)
Exemplary number of scans (minimum, maximum, ranges)	30-999 *excess scans can always be collected and then excluded from analysis (such as skip every other scan) -scans that occur after complete sedimentation of virus can be	30-999	30-999	30-999

	excluded			
Exemplary confidence level of the F statistic	F=0.68	F=0.68	F=0.68	F=0.68
Exemplary ranges for Smin	1-100S	1-100S	1-100S	1-100S
Exemplary ranges for Smax	100-1000S	100-5000S	100-5000S	100-5000S
Exemplary ranges for resolution	*Resolution depends on S Max 200S-1000S	200-5000S	200-5000S	200-5000S
Exemplary frictional ratio	Use the FIT command to determine frictional ratio. Since AAV is ~spherical, in embodiments, 1 may be used as the frictional ratio.	Use FIT command to determine frictional ratio or set at 1.	Use FIT command to determine frictional ratio	Use FIT command to determine frictional ratio
Exemplary ranges for AUC speed	10,000-20,000 rpm	3,000-10,000 rpm	3,000-10,000 rpm	3,000-10,000 rpm
Exemplary absorbances for monitoring sedimentation of viral particles	260nm 280 nm 230 nm	260nm 280 nm 230 nm	260nm 280 nm 230 nm	260nm 280 nm 230 nm
Exemplary methods for monitoring sedimentation of viral particles	IF Absorbance	IF Absorbance	IF Absorbance	IF Absorbance
Radial invariant (RI) and time invariant (TI) noise subtractions, alternative subtractions/calculations	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection
Scanning frequency	*when using absorbance detection system, limited by speed of absorbance scan (~60 Seconds)-scan as fast as system	Scan with no delay through scan with 60 second delay	Scan with no delay through scan with 60 second delay	Scan with no delay through scan with 60 second delay

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	allows with no delay IF only: collect every 10 -60 seconds (10-60 second delay)			
Temperature ranges	4°C -20°C	4°C -20°C	4°C -20°C	4°C -20°C

[0110] In some aspects, the invention provides methods to determine the presence of empty capsids in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peak that corresponds to the S value of empty capsid particles indicates that presence of empty capsid particles. In some embodiments, the invention provides methods of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value corresponding to empty capsid particles is compared to the total amount of all recombinant viral particles in the preparation by integrating all peaks on the plot of C(S) vs. S.

[0111] In some aspects, the invention provides methods to determine the presence of recombinant viral particle variants in a preparation of recombinant viral particles (*e.g.*, rAAV,

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rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peak that corresponds to the S value that differs from the S value of recombinant viral capsid particles comprising a full intact recombinant viral genome indicates that presence of recombinant viral particle variants. In some embodiments, the invention provides methods of measuring the relative amount recombinant viral particle variants in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value that differs from the S value of recombinant viral capsid particles comprising a full intact recombinant viral genome is compared to the total amount of all recombinant viral particles in the preparation by integrating all peaks on the plot of C(S) vs. S. In some embodiments, the recombinant viral particle variants comprise recombinant viral genomes that are smaller (*e.g.*, truncated) or larger than the full length intact viral genome. Other viral-encapsidated DNA impurities can also be detected.

[0112] In some embodiments, the invention provides methods of monitoring the removal of empty capsids and/or recombinant viral particles with variant genomes during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids using AUC as described herein. A decrease in the relative amount of

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empty capsids and/or recombinant viral particles comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

[0113] In some embodiments, the invention provides methods of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant viral particles in the preparation. In some embodiments, the species of recombinant viral particle identified by the methods of the invention include, but are not limited to full recombinant viral particles comprising intact recombinant viral genomes, empty recombinant viral capsid particles, and recombinant viral particles comprising variant recombinant viral genomes. In some embodiments the variant genomes are smaller than the intact recombinant viral genome (*e.g.*, truncated genomes). In some embodiments, the variant genomes are larger than the intact recombinant viral genome (*e.g.*, aggregates, recombinants, *etc.*). In some embodiments the variant genomes include genomes that are smaller and larger than the intact recombinant viral genome.

[0114] In some embodiments, the invention provides methods of monitoring the heterogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the relative amount of full capsids comprising an intact recombinant viral genome, empty capsids and/or recombinant viral particles with variant genomes using AUC as described herein, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

[0115] In embodiments of the embodiments described above, the recombinant viral particles have been purified using one or more purification steps. Examples of purification steps include but are not limited to equilibrium centrifugation, anion exchange filtration, tangential flow

filtration (TFF), apatite chromatography, heat inactivation of helper virus, hydrophobic interaction chromatography, immunoaffinity chromatography, size exclusion chromatography (SEC), nanofiltration, cation exchange chromatography, and anion exchange chromatography.

[0116] In embodiments of the embodiments described above, the recombinant viral particles comprise a self-complementary AAV (scAAV) genome. In some embodiments, the recombinant AAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, a therapeutic transgene coding strand) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the therapeutic transgene) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide sequence along most or all of its length. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR. In some embodiments, the scAAV viral particles comprise a monomeric form of an scAAV genome. In some embodiments, the scAAV viral particles comprise the dimeric form of and scAAV genome. In some embodiments, AUC as described herein is used to detect the presence of rAAV particles comprising the monomeric form of an scAAV genome. In some embodiments, AUC as described herein is used to detect the presence of rAAV particles comprising the dimeric form of an scAAV genome. In some embodiments, the packaging of scAAV genomes into capsid is monitored by AUC described herein.

[0117] In embodiments of the embodiments described above, the rAAV particles comprise an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid (*e.g.*, a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAVrh8R, an AAV9 capsid (*e.g.*, a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV DJ capsid (*e.g.*, an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an

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AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In embodiments of the above embodiments described above, the rAAV particles comprise at least one AAV1 ITR, AAV2 ITR, AAV3 ITR, AAV4 ITR, AAV5 ITR, AAV6 ITR, AAV7 ITR, AAV8 ITR, AAVrh8 ITR, AAV9 ITR, AAV10 ITR, AAVrh10 ITR, AAV11 ITR, AAV12 ITR, AAV DJ ITR, goat AAV ITR, bovine AAV ITR, or mouse AAV ITR. In some embodiments, the rAAV particles comprise ITRs from one AAV serotype and AAV capsid from another serotype. For example, the rAAV particles may comprise a therapeutic transgene flanked by at least one AAV2 ITR encapsidated into an AAV9 capsid. Such combinations may be referred to as pseudotyped rAAV particles.

IV. Viral particles

[0118] The methods disclosed herein may find use, *inter alia,* in characterizing species of interest in a variety of viral particles (*e.g.*, viral particles with a full genome, as compared to viral particles with truncated genomes and/or viral particles comprising DNA impurities).

[0119] In some embodiments, the viral particle is a recombinant AAV particle comprising a nucleic acid comprising a transgene flanked by one or two ITRs. The nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises the protein coding sequence(s) of interest (*e.g.*, a therapeutic transgene) operatively linked components in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette. The expression cassette is flanked on the 5' and 3' end by at least one functional AAV ITR sequences. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson *et al.*, *PNAS*, 2000, 97(7)3428-32; Passini *et al.*, *J. Virol.*, 2003, 77(12):7034-40; and Pechan *et al.*, *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (*e.g.*, as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV

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serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao *et al.*, *PNAS*, 2002, 99(18): 11854-6; Gao *et al.*, *PNAS*, 2003, 100(10):6081-6; and Bossis *et al.*, *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid, or the like. In some embodiments, the nucleic acid in the AAV comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, AAV12 or the like. In further embodiments, the rAAV particle comprises capsid proteins of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAV11, AAV12 or the like. In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, *et al. J. Virol.* 2004, 78(12):6381).

[0120] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle can comprise AAV9 capsid proteins and at least one AAV2 ITR or it can comprise AAV2 capsid proteins and at least one AAV9 ITR. In yet another example, a rAAV particle can comprise capsid proteins from both AAV9 and AAV2, and further comprise at least one AAV2 ITR. Any combination of AAV serotypes for production of a rAAV particle is provided herein as if each combination had been expressly stated herein.

[0121] In some embodiments, the AAV comprises at least one AAV1 ITR and capsid protein from any of AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV2 ITR and capsid protein from any of AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV3 ITR and capsid protein from any of AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV

comprises at least one AAV4 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV5 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV6 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV7 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV8 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV9 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAVrh8 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAVrh10 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV11 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAV9, AAVrh10, and/or AAV12. In some embodiments, the AAV comprises at least one AAV12 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV rh8, AAV9, AAVrh10, and/or AAV11.

Self-complementary AAV viral genomes

[0122] In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome. AAV viral particles with self-complementing genomes and methods of use of self-complementing AAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,765,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., *et al.*, (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (*e.g.*, complementing coding and non-coding strands of a transgene). In some embodiments, the invention provides an AAV

viral particle comprising an AAV genome, wherein the rAAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, a therapeutic transgene coding strand) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the therapeutic transgene) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide sequence along most or all of its length. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR (*e.g.*, the right ITR). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0123] In some embodiments, the viral particle is an adenoviral particle. In some embodiments, the adenoviral particle is a recombinant adenoviral particle, *e.g.*, a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of adenoviral origin) between two ITRs. In some embodiments, the adenoviral particle lacks or contains a defective copy of one or more E1 genes, which renders the adenovirus replication-defective. Adenoviruses include a linear, double-stranded DNA genome within a large (~950Å), non-enveloped icosahedral capsid. Adenoviruses have a large genome that can incorporate more than 30kb of heterologous sequence (*e.g.*, in place of the E1 and/or E3 region), making them uniquely suited for use with larger heterologous genes. They are also known to infect dividing and non-dividing cells and do not naturally integrate into the host genome (although hybrid variants may possess this ability). In some embodiments, the adenoviral vector may be a first generation adenoviral vector with a heterologous sequence in place of E1. In some embodiments, the adenoviral vector may be a second generation adenoviral vector with additional mutations or deletions in E2A, E2B, and/or E4. In some embodiments, the adenoviral vector may be a third generation or gutted adenoviral vector that lacks all viral coding genes, retaining only the ITRs

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and packaging signal and requiring a helper adenovirus *in trans* for replication, and packaging. Adenoviral particles have been investigated for use as vectors for transient transfection of mammalian cells as well as gene therapy vectors. For further description, see, *e.g.*, Danthinne, X. and Imperiale, M.J. (2000) *Gene Ther.* 7:1707-14 and Tatsis, N. and Ertl, H.C. (2004) *Mol. Ther.* 10:616-29.

[0124] In some embodiments, the viral particle is a recombinant adenoviral particle comprising a nucleic acid comprising a transgene. Use of any adenovirus serotype is considered within the scope of the present invention. In some embodiments, the recombinant adenoviral vector is a vector derived from an adenovirus serotype, including without limitation, AdHu2, AdHu 3, AdHu4, AdHu5, AdHu7, AdHu11, AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, and porcine Ad type 3. The adenoviral particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise an adenoviral particle in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped recombinant adenoviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped recombinant adenoviral particles are derived from a foreign virus or from another adenovirus serotype. In some embodiments, the foreign viral capsid proteins are derived from, including without limitation, reovirus type 3. Examples of vector and capsid protein combinations used in pseudotyped adenovirus particles can be found in the following references (Tatsis, N. *et al.* (2004) *Mol. Ther.* 10(4):616-629 and Ahi, Y. *et al.* (2011) *Curr. Gene Ther.* 11(4):307-320). Different adenovirus serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes, include without limitation, lung (*e.g.* HuAd3), spleen and liver (*e.g.* HuAd37), smooth muscle, synoviocytes, dendritic cells, cardiovascular cells, tumor cell lines (*e.g.* HuAd11), and dendritic cells (*e.g.* HuAd5 pseudotyped with *reovirus type 3, HuAd30, or HuAd35*). For further description, see Ahi, Y. *et al.* (2011) *Curr. Gene Ther.* 11(4):307-320, Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40, and Tatsis, N. *et al.* (2004) *Mol. Ther.* 10(4):616-629.

[0125] In some embodiments, the viral particle is a lentiviral particle. In some embodiments, the lentiviral particle is a recombinant lentiviral particle, *e.g.*, a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentiviral origin) between

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two LTRs. Lentiviruses are positive-sense, ssRNA retroviruses with a genome of approximately 10 kb. Lentiviruses are known to integrate into the genome of dividing and non-dividing cells. Lentiviral particles may be produced, for example, by transfecting multiple plasmids (typically the lentiviral genome and the genes required for replication and/or packaging are separated to prevent viral replication) into a packaging cell line, which packages the modified lentiviral genome into lentiviral particles. In some embodiments, a lentiviral particle may refer to a first generation vector that lacks the envelope protein. In some embodiments, a lentiviral particle may refer to a second generation vector that lacks all genes except the gag/pol and tat/rev regions. In some embodiments, a lentiviral particle may refer to a third generation vector that only contains the endogenous rev, gag, and pol genes and has a chimeric LTR for transduction without the tat gene (see Dull, T. *et al.* (1998) *J. Virol.* 72:8463-71). For further description, see Durand, S. and Cimarelli, A. (2011) *Viruses* 3:132-59.

[0126] In some embodiments, the viral particle is a recombinant lentiviral particle comprising a nucleic acid comprising a transgene. Use of any lentiviral vector is considered within the scope of the present invention. In some embodiments, the lentiviral vector is derived from a lentivirus including, without limitation, human immunodeficiency virus-1 (HIV-1), human immunodeficiency virus-2 (HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), Jembrana disease virus (JDV), visna virus (VV), and caprine arthritis encephalitis virus (CAEV). The lentiviral particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise a lentivirus vector in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped recombinant lentiviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped recombinant lentiviral particles are derived from a foreign virus. In some embodiments, the foreign viral capsid protein used in pseudotyped recombinant lentiviral particles is Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped recombinant lentiviral particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped recombinant lentiviral particles. In other embodiments, the foreign viral capsid proteins are derived from, including without limitation, Chandipura virus, Rabies virus, Mokola virus, Lymphocytic choriomeningitis virus (LCMV), Ross River virus (RRV), Sindbis virus, Semliki Forest virus (SFV), Venezuelan equine

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encephalitis virus, Ebola virus Reston, Ebola virus Zaire, Marburg virus, Lassa virus, Avian leukosis virus (ALV), Jaagsiekte sheep retrovirus (JSRV), Moloney Murine leukemia virus (MLV), Gibbon ape leukemia virus (GALV), Feline endogenous retrovirus (RD114), Human T-lymphotropic virus 1 (HTLV-1), Human foamy virus, Maedi-visna virus (MVV), SARS-CoV, Sendai virus, Respiratory syncytia virus (RSV), Human parainfluenza virus type 3, Hepatitis C virus (HCV), Influenza virus, Fowl plague virus (FPV), or Autographa californica multiple nucleopolyhedro virus (AcMNPV). Examples of vector and capsid protein combinations used in pseudotyped Lentivirus particles can be found, for example, in Cronin, J. *et al.* (2005). *Curr. Gene Ther.* 5(4):387-398. Different pseudotyped recombinant lentiviral particles can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). For example, tissues targeted by specific pseudotyped recombinant lentiviral particles, include without limitation, liver (*e.g.* pseudotyped with a VSV-G, LCMV, RRV, or SeV F protein), lung (*e.g.* pseudotyped with an Ebola, Marburg, SeV F and HN, or JSRV protein), pancreatic islet cells (*e.g.* pseudotyped with an LCMV protein), central nervous system (*e.g.* pseudotyped with a VSV-G, LCMV, Rabies, or Mokola protein), retina (*e.g.* pseudotyped with a VSV-G or Mokola protein), monocytes or muscle (*e.g.* pseudotyped with a Mokola or Ebola protein), hematopoietic system (*e.g.* pseudotyped with an RD114 or GALV protein), or cancer cells (*e.g.* pseudotyped with a GALV or LCMV protein). For further description, see Cronin, J. *et al.* (2005). *Curr. Gene Ther.* 5(4):387-398 and Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40.

[0127] In some embodiments, the viral particle is a herpes simplex virus (HSV) particle. In some embodiments, the HSV particle is a rHSV particle, *e.g.*, a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentiviral origin) between two TRs. HSV is an enveloped, double-stranded DNA virus with a genome of approximately 152 kb. Advantageously, approximately half of its genes are nonessential and may be deleted to accommodate heterologous sequence. HSV particles infect non-dividing cells. In addition, they naturally establish latency in neurons, travel by retrograde transport, and can be transferred across synapses, making them advantageous for transfection of neurons and/or gene therapy approaches involving the nervous system. In some embodiments, the HSV particle may be replication-defective or replication-competent (*e.g.*, competent for a single replication cycle through

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inactivation of one or more late genes). For further description, see Manservigi, R. *et al.* (2010) *Open Virol. J.* 4:123-56.

[0128] In some embodiments, the viral particle is a rHSV particle comprising a nucleic acid comprising a transgene. Use of any HSV vector is considered within the scope of the present invention. In some embodiments, the HSV vector is derived from a HSV serotype, including without limitation, HSV-1 and HSV-2. The HSV particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise a HSV vector in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped rHSV particles. In some embodiments, foreign viral capsid proteins used in pseudotyped rHSV particles are derived from a foreign virus or from another HSV serotype. In some embodiments, the foreign viral capsid protein used in a pseudotyped rHSV particle is a Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped rHSV particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped rHSV particles. In other embodiments, the foreign viral capsid protein may be from a different HSV serotype. For example, an HSV-1 vector may contain one or more HSV-2 capsid proteins. Different HSV serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes include without limitation, central nervous system and neurons (*e.g.* HSV-1). For further description, see Manservigi, R. *et al.* (2010) *Open Virol J* 4:123-156, Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40, and Meignier, B. *et al.* (1987) *J. Infect. Dis.* 155(5):921-930.

V. Production of Viral Vectors

[0129] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids. rAAV production cultures for the production of rAAV virus particles all require; 1) suitable host cells, including, for example, human-derived cell lines such as HeLa, A549, or 293 cells, or insect-derived cell lines such as SF-9, in the case of baculovirus production systems; 2) suitable helper virus function, provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus,

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baculovirus, or a plasmid construct providing helper functions; 3) AAV rep and cap genes and gene products; 4) a transgene (such as a therapeutic transgene) flanked by at least one AAV ITR sequences ; and 5) suitable media and media components to support rAAV production. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (e.g., Spodoptera frugiperda (Sf9) cells).

[0130] Suitable rAAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, rAAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of rAAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of rAAV in production cultures.

[0131] In some aspects, the invention provides methods for preparing rAAV particles with reduced empty capsids comprising a) culturing host cells under conditions suitable for rAAV production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release rAAV particles; c) isolating the rAAV particles produced by the host cell; and d) analyzing the rAAV particles for the presence of empty capsids

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and/or rAAV particles with variant genomes by analytical ultracentrifugation as described above. In some embodiments, the p5 promoter of the nucleic acid encoding AAV rep and cap regions is located 3' to the rep and/or cap coding region. In some embodiments, the nucleic acid encoding AAV rep and cap coding regions is plasmid pHLP, pHLP19, or pHLP09 (see US Patent Nos. 5,622,856; 6,001,650; 6,027,931; 6,365,403; 6,376,237; and 7,037,713; the content of each is incorporated herein in its entirety). In some embodiments, the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

[0132] rAAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, rAAV production cultures include attachment- dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. rAAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0133] rAAV vector particles of the invention may be harvested from rAAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of rAAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0134] Numerous methods are known in the art for production of adenoviral vector particles. For example, for a gutted adenoviral vector, the adenoviral vector genome and a helper adenovirus genome may be transfected into a packaging cell line (*e.g.*, a 293 cell line). In some embodiments, the helper adenovirus genome may contain recombination sites flanking its packaging signal, and both genomes may be transfected into a packaging cell line that expresses a recombinase (*e.g.*, the Cre/loxP system may be used), such that the adenoviral vector of interest is packaged more efficiently than the helper adenovirus (see, *e.g.*, Alba, R. *et al.* (2005) *Gene Ther.*

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12 Suppl 1:S18-27). Adenoviral vectors may be harvested and purified using standard methods, such as those described herein.

[0135] Numerous methods are known in the art for production of lentiviral vector particles. For example, for a third-generation lentiviral vector, a vector containing the lentiviral genome of interest with gag and pol genes may be co-transfected into a packaging cell line (*e.g.,* a 293 cell line) along with a vector containing a rev gene. The lentiviral genome of interest also contains a chimeric LTR that promotes transcription in the absence of Tat (see Dull, T. *et al.* (1998) *J. Virol.* 72:8463-71). Lentiviral vectors may be harvested and purified using methods (*e.g.*, Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011) described herein.

[0136] Numerous methods are known in the art for production of HSV particles. HSV vectors may be harvested and purified using standard methods, such as those described herein. For example, for a replication-defective HSV vector, an HSV genome of interest that lacks all of the immediate early (IE) genes may be transfected into a complementing cell line that provides genes required for virus production, such as ICP4, ICP27, and ICP0 (see, *e.g.,* Samaniego, L.A. *et al.* (1998) *J. Virol.* 72:3307-20). HSV vectors may be harvested and purified using methods described (*e.g.*, Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79).

VI. Purification of rAAV Vectors

[0137] At harvest, rAAV production cultures of the present invention may contain one or more of the following: (1) host cell proteins; (2) host cell DNA; (3) plasmid DNA; (4) helper virus; (5) helper virus proteins; (6) helper virus DNA; and (7) media components including, for example, serum proteins, amino acids, transferrins and other low molecular weight proteins. In addition, rAAV production cultures further include rAAV particles having an AAV capsid serotype selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAV9, AAV10, AAVrh10, AAV11, or AAV12. In some embodiments, the rAAV production cultures further comprise empty AAV capsids (*e.g.,* a rAAV particle comprising capsid proteins but no rAAV genome). In some embodiments, the rAAV production cultures further comprise rAAV particles comprising variant rAAV genomes (*e.g.,* a rAAV particle comprising a rAAV genome that differs from an intact full-length rAAV genome). In

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some embodiments, the rAAV production cultures further comprise rAAV particles comprising truncated rAAV genomes. In some embodiments, the rAAV production cultures further comprise rAAV particles comprising AAV-encapsidated DNA impurities.

[0138] In some embodiments, the rAAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0139] In some embodiments, the rAAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0140] rAAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the rAAV particles; rAAV capture by apatite chromatography; heat inactivation of helper virus; rAAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and rAAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify rAAV particles are found, for example, in Xiao *et al.*, (1998) *Journal of Virology* 72:2224-2232; US Patent Numbers 6,989,264 and 8,137,948 and WO 2010/148143. Methods to purify adenovirus particles are found, for example, in Bo, H *et al.*, (2014) *Eur. J. Pharm. Sci.* 67C:119-125. Methods to purify lentivirus particles are found, for example, in Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for example, in Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79.

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EXAMPLES

[0141] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Characterization of recombinant adeno-associated viral vector preparations by analytical ultracentrifugation

[0142] Adeno-associated viruses (AAV) have features that make them attractive as vectors for gene therapy. Wild-type AAV consists of two open reading frames (rep and cap) which code for all structural and regulatory elements required for assembly, replication and infection. The rep ORF codes for Rep 78 and 68 proteins which have genome replication functions as well as Rep 52 and 40 proteins which are involved in single strand replication and packaging. The cap ORF codes for the three structural capsid proteins: VP1, VP2 and VP3. Recombinant AAV vector is typically produced by the triple transfection method using the "gutless" vector approach (Xiao, X, *et al.*, 1998, *J. Virol.* 3:2224-2232). The rep and cap genes are replaced with the therapeutic gene and its regulatory elements sandwiched between a 5' and 3' inverted terminal repeat (ITR), the rep and cap genes are provided in trans on a separate plasmid and a third plasmid contributes required adenoviral helper genes. It is postulated that the viral capsids are fully assembled and the ITR flanked vector genome is then inserted into the capsid via a capsid pore (Myers, MW & Carter, BJ, 1980, *Virology*, 102:71-82). The resulting population of capsids contains both non-genome containing capsids (empty capsids) as well as genome containing capsids. In addition, capsids may contain incomplete portions of the recombinant viral genome. The vector prep may then purified by affinity chromatography to isolate the capsids from the cellular debris and can be further processed to enrich for intact vector by anion exchange chromatography.

[0143] Based on their recent approval for use in gene therapy, adeno-associated viral (AAV) vectors have emerged as an important class of novel biopharmaceutical drug products. The generation of AAV vector products requires an analytical method that monitors product quality

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with regard to homogeneity, purity, and consistency of manufacturing, yet to date no method to support AAV vector characterization has been established. To meet this demand, the potential use of analytical ultracentrifugation (AUC) as a technique to characterize the homogeneity of AAV vectors was investigated.

Methods

Sample preparation

[0144] In order to support accurate AUC assessment, vector product (AAV2-transgene 2) was highly purified, suitably buffered, and concentrated to greater than 5 x 10^{11} vg/mL. To achieve this, cell supernatants runs were purified using AVB affinity chromatography (GE Healthcare) and buffer-exchanged into PBS, pH 7.2 using a 10K MWCO Slide-a-Lyzer (Thermo Scientific). Product concentration was determined by optical density measurement at 260nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260nm from 0.1 to 1.0, either by direct dilution with PBS or further concentration using Amicon Ultra-0.5 /30K MWCO Centrifugal Filter Device.

Sedimentation velocity AUC data acquisition

[0145] Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis was performed using a ProteomeLab™ XL-I (Beckman Coulter). 400 µL sample was loaded into the sample sector of a two sector velocity cell, and 400 µL PBS was loaded into the corresponding reference sector. The sample was placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of 20°C and full vacuum were maintained for one hour. Sedimentation velocity centrifugation was performed at 20,000 RPM, 20°C, 0.003 cm radial step setting, with no delay and with no replicates. Absorbance (260 nm) and Raleigh interference optics were used to simultaneously record radial concentration as a function of time until the smallest sedimenting component cleared the optical window (1.2 hour). Assay throughput was limited to a single sample per run based on absorbance scan collection times of greater than one minute, as well as the large size and rapid sedimentation of AAV.

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AUC data analysis

[0146] The percent full capsid was determined by analyzing approximately 75 scans from each detection method using the SEDFIT (NIH/see worldwide web at analyticalultracentrifugation.com) continuous size C(S) distribution model. Second (2nd) derivative regularization was applied to the fitting with a confidence level of F statistic=0.68. The following C(S) parameters were held constant: resolution=200S, S min=1, S max=200 and frictional ratio=1.0. RI and TI noise subtractions were applied, and the meniscus position was allowed to float, letting the software choose the optimal position. This model fit the data to the Lamm equation, and the resulting size distribution was a "distribution of sedimentation coefficients" that looked like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) were determined for each component in the distribution. Each AUC run was an independent assay, and each analysis was monitored for the following attributes to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\,nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans.

Absorbance optics (260 nm)

[0147] Extinction coefficients were used to calculate molar concentration and the actual percent value of the intact vector peak from absorbance data. Molar absorbance extinction coefficients for both empty capsids ($\epsilon_{260}/_{capsid}$=3.72e6) and intact vector ($\epsilon_{260}/_{vector}$=3.00e7) were calculated based on published formulae (Sommer *et al.* (2003) *Mol Ther.*, 7:122-8). Extinction coefficients were available for empty capsid and intact vector peaks. The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.*, 78:1606-19. Molar concentration of both intact vector and empty capsid were calculated using Beer's Law, and the percentage of full capsid was calculated from these values. Values were reported in terms of the percentage of full capsid.

Generation of an AUC standard curve

[0148] Because it is not possible to determine empirically the extinction coefficient of fragmented genomes of unknown size and sequence, a relationship between S value and genome

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size was established. To achieve this, rAAV vector preps with encapsidated viral genomes of known nucleotide size were analyzed by AUC, and a corresponding S value was determined as described above.

Production of rAAV by transient transfection

[0149] Recombinant AAV vector was produced by the triple transfection method using the "gutless" vector approach (Xiao *et al.* (1998) *J. Virol.*, 3:2224-32). In this approach, the rep and cap genes were replaced with the therapeutic gene and its regulatory elements, both sandwiched between a 5' and 3' inverted terminal repeat (ITR). The rep and cap genes were provided in trans on a separate plasmid, and a third plasmid contributed the required adenoviral helper genes. Without wishing to be bound to theory, it is postulated that the viral capsids are fully assembled, and the ITR flanked vector genome is then inserted into the capsid via a capsid pore (Myers & Carter (1980) *Virology*, 102:71-82). The resulting population of capsids contained both non-genome-containing capsids (empty capsids) and genome-containing capsids.

Production of rAAV by producer cell platform

[0150] The AAV producer cell line is an alternative production platform used to generate clinical rAAV vectors. With this method, a HeLa S3 cell, adapted to growth in suspension, was engineered to have integrated copies of the AAV rep and cap genes required for vector replication and packaging, in addition to vector sequences and the selectable marker (*see, e.g.*, Puro: Thorne *et al.* (2009) *Hum. Gene Ther.*, 20:707-14). Once infected with WT Adenovirus, which provides the helper functions required for replication, the cell produced recombinant AAV vector as well as adenovirus, which was subsequently removed during the purification process using ion-exchange chromatography.

Other methods

[0151] Synthetic transgenes were cloned into a plasmid that contained a promoter of choice and bovine growth hormone polyadenylation signal sequence (polyA). The entire transgene expression cassette was then cloned into previral plasmid vector pAAVDC64 containing AAV2 inverted terminal repeats. The total size of the resulting AAV genomes in the respective expression plasmids (including the region flanked by ITRs) was 4 - 4.6 kb. The recombinant

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vectors were produced by triple transfection of 293 cells using helper plasmids expressing rep2/cap sequences and Adenovirus helper functions, pAd Helper (Stratagene, La Jolla, CA USA) The rep/cap helper expressed rep from AAV serotype 2, while the cap sequence encoded one of the following sequences: AAV cap 1, 2, 5, 9, or rh8R. Vectors were purified by affinity chromatography and in some cases were further purified to remove empty particles (see, *e.g.*, Qu *et al.* (2007) J. *Virol. Methods.* 140:183-92).

Results

[0152] Analytical ultracentrifugation (AUC) using classical boundary sedimentation velocity was used to reveal the particle heterogeneities of recombinant adeno-associated virus (rAAV) vector preps. A mixture containing 20% rAAV2 particles with the full genome and 80% empty capsids was created by mixing together purified empty capsids and purified genome-containing capsids at defined ratios. The empty and full capsids were generated by $CsCl_2$ gradient purification of a mixture of empty and full capsids following triple transfection production. To monitor the movement of rAAV2 particles in response to a centrifugal force, this mixture of rAAV2 capsids was scanned at an absorbance of 260nm along a centrifugal field at defined time intervals. **FIG. 1A** shows a representative scanning profile following centrifugation of the AAV2 mixture at 20,000 rpm for 1.2 hrs (until the smallest sedimenting species cleared the optical window). Scans represented the acquisition of concentration data as a function of radius *r*, at times *t*, to yield a series of concentration scans that revealed the complete migration pattern of constituent vector particles in the rAAV2 vector prep. In these sigmoidal curves or boundaries, the leading edge of the curve represented the faster sedimenting species (*i.e.*, the genome-containing rAAV2 capsid), and the trailing edge of the curve represented the slower sedimenting species (*i.e.*, the "empty" rAAV2 capsids) (**FIG. 1A**).

[0153] Plotting the differential sedimentation coefficient distribution value, C(S), versus the sedimentation coefficient (in Svedberg units, S) yielded distinct peaks with unique sedimentation coefficients for both the empty and genome-containing capsid species (**FIG. 1B**). The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.*, 78:1606-19. In order to calculate molar concentrations and percent value for each capsid species from the absorbance data, extinction coefficients were used according to Table 2.

Table 2. Extinction coefficients and molar concentrations for capsid species

Species	Signal (Abs $_{260nm}$)	ε_{260nm}	Molar concentration (M)	Relative abundance (%)
Peak 1	0.0479	3.73E+06	1.28E-08	79
Peak 2	0.0909	2.59E+07	3.51E-09	21
Sum			1.63E-08	

[0154] Molar absorbance extinction coefficients for both the empty capsid ($\varepsilon_{260}/_{capsid}$ = 3.72e6) and genome containing capsid ($\varepsilon_{260}/_{capsid}$ = 3e7) were calculated using genome size and published formulae (Sommer *et al.*, 2003). Molar concentrations of both genome-containing and empty capsids were then calculated using Beer's Law. The molar concentration of each species was used to calculate its relative abundance, expressed as a percentage of total capsids (**FIG. 1**). These results demonstrated that AUC may be used to accurately distinguish and quantify empty capsids and genome-containing capsids from a heterogeneous vector preparation.

[0155] For the rAAV2 vector prep shown in **FIG. 1**, capsids containing a full genome were represented by the peak sedimenting at 94S and accounted for 21% of the vector prep. Empty capsids sedimented with an S value of 64S and accounted for 79% of the vector prep. These sedimentation coefficient values were confirmed by AUC analysis of pure populations of empty (**FIG. 2A**) or genome containing particles (**FIG. 2B**). The AUC profile of a pure population of rAAV2 empty capsids revealed a single peak with a sedimentation coefficient of 64S, whereas the AUC profile of a pure population of rAAV2AUC genome-containing capsids revealed a single peak with a higher sedimentation coefficient of 94S. These results agreed with the values generated from a heterogeneous preparation and further confirmed that AUC methods may be used to quantify genome-containing and empty AAV capsids from a heterogeneous preparation containing both species.

[0156] The AUC method was further assessed for reproducibility by performing five independent AUC runs of the same vector sample (scAAV2/9 LP2), as shown in Table 3. The sedimentation coefficients for both genome-containing and empty AAV2 capsids were highly reproducible, yielding coefficients of variation from 0.5-0.6%. Similarly, the relative abundance (expressed as a percentage of the total) of genome-containing capsids was determined with a coefficient of variation of approximately 2%. These results indicated that the AUC method for

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quantifying genome-containing and empty AAV2 capsids yields highly reproducible and consistent values.

Table 3. Five independent assays on scAAV2/9 LP2 sample.

AUC Run	Empty Capsid, Peak 1 (S)	Full Capsid, Peak 3	
		% Full Capsids	S
20110927A	64.3	34.1	84.0
20111003A	64.6	34.2	84.6
20111005A	65.1	35.0	84.5
20111005B	64.2	35.2	83.8
20111011A	64.1	33.5	84.5
Mean	64.5	34.4	84.3
Standard Deviation	0.4	0.7	0.4
% CV	0.6	2.1	0.5

Example 2: Comparison of interference and absorbance detection methods for AUC

[0157] An alternative optical detection method for AUC, Rayleigh Interference Optics, was also evaluated. This detection method measures the sample concentration based on refractive index differences between a reference solution and the AAV containing sample. Like absorbance detection, interference detection can be applied to any rAAV regardless of the sequence of the genome. Unlike absorbance detection, which requires an extinction coefficient, interference detection yields integrated peaks that are directly proportional to concentration.

[0158] Pure populations of empty and genome-containing AAV2 capsids were mixed at a 1:1 ratio and analyzed by AUC using both interference (**FIG. 3A**) and absorbance detection methods (**FIG. 3B**). Interference detection revealed two populations of AAV capsids at the approximate expected ratios of 43% empty and 57% genome-containing (**FIG. 3A**). Both detection methods yielded similar abundance ratios. However, comparing the peak sizes generated by both methods illustrates the disconnect between peak height and concentration with absorbance detection (compare size of "empty capsid" peaks in **FIG. 3A** and **3B**). The data generated by both methods are compared in Table 4. The ratio of absorbance signal to interference signal (A_{260nm}/IF) can be used in a fashion analogous to the 260/280 ratio of absorbance data, and this assisted in identifying peaks in the C(S) distribution.

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Table 4. S values and relative abundance generated by absorbance and interference detection.

Peak	Absorbance (260 nm)		Interference			A_{260nm}/IF
	Sedimentation coefficient (S)	Relative abundance (%)	Sedimentation coefficient (S)	Signal (fringes)	Relative abundance (%)	
Peak 1	63	47	62	0.080	43	0.41
Peak 2	93	53	92	0.104	57	2.38
Sum				0.184		

[0159] Although interference optics offers precision and resolution, it may require a high concentration of sample. Moreover, interference optics may be affected by a mismatch between the reference and AAV sample buffer. AAV samples, however, typically contain a low protein concentration, and it may be necessary to completely match the AAV sample and reference buffers.

Example 3: Influence of production method on AAV vector heterogeneity

[0160] The previous examples demonstrated that the AUC method is a highly accurate and reproducible way to resolve and quantify empty and genome-containing AAV capsids from a heterogeneous mixture. This capability could be advantageous for a variety of applications to evaluate the quality of AAV vector preparations. For example, a major problem in producing pure AAV vector preparations is the presence of capsids with partial or fragmented genomes. To illustrate the utility of the AUC method for resolving these species, AAV vectors generated by two different methods, termed the "triple transfection" and "producer cell line" methods, were analyzed by AUC.

[0161] An AAV2 vector harboring the transgene 2 was produced using either the triple transfection method (**FIG. 4**) or the producer cell line method (**FIG. 5**). For a description of these methods, see Example 1. Following chromatographic purification, both vector preps were analyzed by AUC. **FIG. 6A** shows a schematic of this AAV2 vector genome.

[0162] The AUC profiles of vector preps produced by these methods were remarkably different. Using the producer cell line method, 74% of capsids contained a full genome, represented by the 92S species (**FIG. 6B**). 19% were empty capsids, with the remainder containing a fragmented genome (75S species, 7%). In contrast, 82% of the capsids produced by

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the triple transfection method were empty, 64S species (**FIG. 6C**), with 11% of capsids containing a fragmented genome (76S and 84S species) and only 8% of capsids having a full genome (94S).

[0163] These results demonstrate that vector preparations generated using the producer cell line technology may have high quality, containing predominantly capsids with a full genome. The vast majority of capsids produced by the triple transfection method are empty, with a greater proportion of capsids having a fragmented genome. These results also highlight the ability of the AUC method to resolve capsids with fragmented genomes, in addition to full genome-containing and empty capsids. Moreover, they illustrate the power of the AUC method in evaluating the quality and homogeneity of vector preparations.

Example 4: Use of AUC to assess removal of empty capsids from vector preps

[0164] The AUC method was evaluated as a tool to monitor removal of empty capsids using chromatographic methods (for methods, see Qu *et al.* (2007) *J. Virol. Methods,* 140:183-92). Separation of empty and genome-containing capsids was performed using anion exchange chromatography (**FIG. 7A**). AUC was performed on the resolved peaks to demonstrate that the genome-containing rAAV2 particles were enriched in the later fractions eluted from the resin ("Full Genome Capsid" in **FIG. 7A**).

[0165] As shown in **FIG. 7B**, AUC analysis revealed that genome-containing capsids represented 94% of the vector prep upon elution from the column. This later fraction yielded a single peak with a sedimentation coefficient of 92S. In contrast, the rAAV2 vector prep prior to the chromatographic step (**FIG. 7C**) had a substantial level of empty capsids. AUC analysis revealed two peaks with S values of 63 and 93, with the 63S peak (empty capsid) representing 52% of the total capsid population. These results show that chromatographic methods are highly effective in removing empty capsids from AAV vector preparations. Importantly, they demonstrate the utility of applying the AUC method to evaluate vector quality upon purification. The AUC method is a useful tool for evaluating different vector purification protocols or techniques.

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Example 5: Assessment of viral genome integrity by the AUC method

[0166] As illustrated in Example 3, AAV vector preparations may contain capsids packaged with fragmented genomes, in addition to full genome and empty species. A major problem in generating homogeneous AAV preparations for therapeutic or research applications is the presence of capsids with fragmented genomes, which may result in aberrant or absent expression of transgenes of interest. Indeed, heterogeneity associated with AAV vector preps has been reported to result from packaging of fragmented genomes or AAV-encapsidated DNA impurities. (Kapranov *et al.* (2012) *Hum. Gene Ther.*, 23:46-55). Therefore, AUC was evaluated as a tool to quantify the aberrant packaging of fragmented genomes in rAAV vector preps.

[0167] Because it is not possible to determine empirically the extinction coefficient of fragmented genomes of unknown size and sequence, a relationship between S value and genome size was established. To achieve this, rAAV vector preps with encapsidated viral genomes of known size were analyzed by AUC, and their corresponding S values were determined, as shown in Table 5. A standard curve was then generated to correlate genome size and S value (**FIG. 8**). This demonstrated a highly linear relationship (R^2=0.9978) between sedimentation coefficient and genome size.

Table 5. S values for rAAV vectors with known genome size.

Predicted Trend Line		Calculated values	
Sedimentation coefficient (S)	**Genome size (# NT)**	**MW**	**Extinction coefficient (260 nm)**
(y)	(x)		
Empty capsid	N/A		3.72E+06
74	880	2.7E+05	9.17E+06
78	1421	4.4E+05	1.25E+07
82	1961	6.1E+05	1.59E+07
84	2232	6.9E+05	1.75E+07
88	2772	8.6E+05	2.09E+07
92	3313	1.0E+06	2.42E+07
96	3853	1.2E+06	2.76E+07
100	4394	1.4E+06	3.09E+07
104	4934	1.5E+06	3.43E+07
108	5475	1.7E+06	3.76E+07

[0168] To demonstrate the utility of AUC to detect genome fragments, a self-complementary vector comprising AAV2 ITRS, a minimal CBA promoter, and an EGFP transgene was packaged

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into an AAV9 capsid (AAV2/9minCBAEGFP; see schematic in **FIG. 9A**). The vector particles were purified to eliminate empty capsids and analyzed by AUC. The standard curve was then used to assign genome size to each of the resolved genome containing capsids. Approximately 25% of the vector prep sedimented as a 101S species, representing an encapsidated genome of ~ 4.3kb (**FIG. 9A**). This 101S peak represented the double stranded dimeric vector genome, which has a predicted size of ~4.3kb. However, the majority of the vector prep (75%), sedimented with an S value of 82, which corresponds to a vector genome size of ~ 2kb (**FIG. 9A**), consistent with packaging of the single stranded monomer. The packaging of monomeric genomes with self-complementary vectors is well documented and is often a result of inadvertent terminal resolution at spurious "trs like" sequences despite the presence of an ITR with a mutated D sequence (McCarty *et al.* (2001) *Gene Ther.*, 8:1248-54).

[0169] **FIG. 9B** shows an alkaline Southern blot of the same vector, scAAV9 EGFP, which revealed two vector populations with genomes ~ 4.3kb and ~2kb in size, corroborating the AUC data in **FIG. 9A**. The Southern blot also confirmed that the monomeric viral genome was preferentially packaged over the dimeric genome. Interestingly, AUC analysis of a single stranded AAV9 EGFP vector (~4kb) revealed a single predominant peak with a measured S value of 99S, corresponding to approximately 4.1kb by the standard curve and 84% of capsid abundance (**FIG. 9C**). These results suggest that single stranded AAV vectors may be packaged in a more homogeneous manner than double stranded vectors. Again, in agreement with the AUC method, Southern blot analysis of this vector prep revealed homogeneous encapsidation of a viral genome of the predicted size of ~4kb (lane 2, **FIG. 9B**). These results demonstrate that the AUC method may be used to measure the size of AAV vector genomes, yielding genome size data in agreement with the standard Southern blotting technique. Using the AUC method, single stranded AAV vectors were found to produce more homogeneous vector preparations than double stranded ones. These results show that the AUC method is a powerful tool to identify and quantify capsid species with incomplete genomes from vector preparations.

Example 6: Use of AUC to assess factors that influence packaging of vector genomes

[0170] The AUC method was next used as a tool to identify factors that influence the packaging of intact AAV vector genomes.

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[0171] As discussed in Example 3, the production of rAAV vectors by transient transfection methods requires the use of three plasmids including a rep/cap helper, an ITR vector plasmid, and a pAd helper (see **FIG. 4**). AUC was used to assess the effect of the rep/cap helper on vector genome packaging for both single stranded and self-complementary AAV vectors. First, a self-complementary AAV vector harboring an EGFP transgene (**FIG. 10A**) was produced using one of two methods. In the first method (**FIG. 10B**), a helper plasmid was used in which rep 78/68 expression was driven by the endogenous p5 promoter ("WT Rep" construct). In the second method (**FIG. 10C**), the helper was modified such that 78/68 expression was reduced by moving the p5 promoter downstream of the cap2 sequence as well as mutating the TATA box ("pHLP Rep" construct). The full scAAV2 EFGP capsid was predicted to have a sedimentation coefficient of 100S in dimeric genome form and 80S in monomeric genome form (**FIG. 10A**).

[0172] AUC analysis of these scAAV2EGFP vector preps revealed a significant difference in vector genome packaging. In the presence of reduced rep78/68 (pHLP), more than half (55%) of the vector prep contained dimeric genomes, represented by the 100S species (**FIG. 10C**). This was the expected sedimentation coefficient for a capsid containing a dimeric genome of 4.4kb. In contrast, the scAAV2EGFP prep generated with the full complement of rep78/68 had significantly less packaged dimeric genomes (26%), with the majority of the capsids containing monomeric genomes and sedimenting at 80S (**FIG. 10B**). These results uncovered a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid, leading to reduced rep 78/68 protein levels.

[0173] A single stranded AAV5 Factor IX vector, AAV5FIX, (**FIG. 11A-B**) and a single stranded AAV5hSMN vector (**FIG. 11C-D**) were generated using rep/cap helpers that differed in rep expression as described above, but cap sequences of AAV2 were replaced by cap sequences of AAV5. Based on the nucleotide size of the FIX expression cassette (4.3kb), the predicted sedimentation coefficient for the AAV5 FIX vector capsid was approximately 101S. AUC analysis of AAV2/5FIX made in the presence of reduced rep78/68 ("pHLP19 Rep") revealed a homogenous profile with the majority of the vector (90%) sedimenting with an S value of the expected size, ~101S (**FIG. 11A**). In contrast, AAV5 FIX vector generated using a rep/cap5 helper expressing wild-type levels of rep 78/68 proteins ("WT Rep") generated a strikingly different AUC profile (**FIG. 11B**). Instead of a predominant peak at 101S, this profile revealed more capsid heterogeneity, with the majority of the AAV5 FIX (80%) sedimenting at a lower S

value of 86S, likely representing packaging of a fragmented genome. Moreover, in this vector sample only 15% of the AAV5 FIX vector capsids sedimented at the correct S value of ~ 104S (**FIG. 11B**).

[0174] AAV5SMN vectors made using these same wild-type and mutated p5 rep/cap helpers also had strikingly different AUC profiles. As seen with the single stranded AAV5FIX vectors, AAV5 SMN vectors generated in the presence of reduced rep78/68 showed less heterogeneity by AUC analysis, with a single capsid species sedimenting at the S value of 101S, consistent with packaging of a genome of the predicted size of ~ 4.4kb (**FIG. 11C**). In contrast, the AUC profile for the same vector genome packaged using "wild-type" levels of rep78/68 protein revealed three distinct AAV vector species, with sedimentation coefficients of 100S (predicted S value for the full vector genome of 4400nt), 92S (representing a fragmented genome of approximately 3300nt) and 80S (representing a fragmented genome of 2000 nucleotides) (**FIG. 11D**). These results confirm a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid using two additional AAV vectors.

[0175] Further analysis of the AAV5SMN and AAV5FIX vector preps was performed by Southern blot analysis of vector DNA. In agreement with the AUC method, Southern blot analysis of AAV5SMN generated with wild-type rep78/68 protein levels revealed packaging of full length (4.4kb) and fragmented (less than 4.4 kb) SMN genomes (**FIG. 12A**, lane 1). In contrast, the AAV5SMN vector generated in the presence of reduced rep78/68 protein contained largely capsids with a full length SMN genome (**FIG. 12A**, lane 2). Interestingly, a comparison of the two AAV5FIX vector preps by Southern analysis revealed the presence of a FIX full length genome even when vector was produced in the presence of wild-type levels of rep 78/68 (**FIG. 12B**, lane 2). However, AUC analysis of this AAV5FIX vector (**FIG. 11B**) showed that 80% of the capsids contained a fragmented genome (~ 3000 nucleotides), which were undetected by the FIX probe.

[0176] Further analysis of the FIX vector preps generated under the two experimental conditions was performed by generating probes to discrete regions of the vector plasmid, including regions of the backbone. A map of this vector is provided in **FIG. 13**. **FIG. 14** shows Southern blotting analyses using these probes to compare these FIX vector preps generated under different conditions. As shown in **FIG. 14A**, both vector preparations (pHLP rep, lane 1; WT rep,

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lane 2) contained the hFIX transgene. However, **FIG. 14B** lane 2confirms that the vector genome species sedimenting at 86S observed in the WT Rep preparation (**FIG. 11B**) was a ~ 3kb fragment. Moreover, this species reacted with an Amp^R specific probe (**FIG. 14B**, lane 2), suggesting that packaging upstream of the 5'ITR had occurred in a rep dependent manner. In contrast, there was no evidence of an Amp^R containing fragment in the rAAV5 FIX vector preps that were generated in the presence of reduced levels of rep 68 / 78 (**FIG. 14B**, lane 1).

[0177] DNA impurities in the AAV FIX preps were also assessed by Q-PCR using primers and probes specific for Amp^R. By Q-PCR, approximately 35% Amp^R titer was detected in AAV5FIX vector preps generated in the presence of "wt" rep, in contrast to less than 1% when the same vector plasmid was used to generate AAV5FIX vector in the presence of reduced rep68/78 (data not shown). These results underscore the utility of AUC analysis for revealing the presence of packaged genomes that would otherwise go undetected by gene specific Southern blot analysis.

[0178] The packaging capacity of AAV vectors has been studied extensively, and although numerous reports have demonstrated successful transduction with vectors packaging oversized AAV genomes, the latter have been shown to be fragmented into subgenomic – length DNA. To further explore the applicability of the AUC method, the heterogeneity of AAV vectors produced using oversized genomes was evaluated. An expression cassette harboring the full length CBA promoter driving expression of the β-phosphodiesterase transgene was packaged as an oversized genome of 5.4kb (**FIG. 15A**) or as a wild type size genome of 4.6kb (**FIG. 15B**). To generate the 4.6kb genome, the CBA promoter was truncated by reducing the size of the intron as previously reported (Gray, SJ *et al.*, (2011) *Hum. Gene Ther.* 22(9):1143-1153).

[0179] As shown in **FIG. 15A**, the AUC profile of the AAV vector prep generated using the oversized vector genome demonstrated that nearly half of the vector prep sedimented as a 93S species, consistent with packaging a fragmented vector genome of approximately 3.5kb. 30% of the preparation was represented by another sub-genomic vector species of approximately 4.9kb sedimenting at 105S. There was no evidence of packaging of a full-length 5.4kb genome, which was predicted to sediment at 108-109S. In contrast, AUC analysis revealed that the same transgene under control of the abbreviated CBA promoter sedimented predominantly as a vector species of 102S, consistent with packaging of the predicted, full-length vector genome of 4.6 kb (**FIG. 15B**). These results demonstrate the utility of AUC analysis in profiling AAV vectors with

oversized genomes, and this profiling is critical, given the observed incidence of genome fragmentation.

[0180] This example demonstrated that the AUC method is highly effective in analyzing the genome size of AAV vector capsids in a heterogeneous preparation. By resolving genome-containing capsids by size (*e.g.*, dimeric and monomeric genomes, or partial fragments thereof), the AUC method represents a powerful tool for assaying the quality of AAV vector preps produced under different conditions. Moreover, the results from three distinct vector systems demonstrated that the AUC method is widely useful for quality control and optimization of conditions to yield improved AAV vector preparations. Importantly, the AUC method is able to detect fragmented genomes that are not detectable by Southern blot analysis. Whereas Southern blotting relies on the presence of DNA probe sequence for detection, the AUC method is sequence-independent. The AUC method has also been demonstrated to be an effective tool in analyzing oversized AAV genomes. In total, these results demonstrate the highly advantageous and effective implementation of AUC methods to analyze multiple types of AAV vector preparations, which have been found to display dramatically variable effects on genome packaging.

Example 7: Characterization of recombinant adenoviral vector preparations by analytical ultracentrifugation

[0181] Adenovirus (Ad) vectors have features that make them attractive as vectors for gene therapy. The generation of Ad vector products requires an analytical method that monitors product quality with regard to homogeneity, purity, and consistency of manufacturing. To meet this demand, the potential use of analytical ultracentrifugation (AUC) as a technique to characterize the homogeneity of Ad vectors was investigated.

Methods

Sample preparation

[0182] In order to support accurate AUC assessment, a recombinant adenovirus serotype 2 vector (Ad2) was prepared and highly purified ty CsCl gradient ultrafiltration to enrich for genome containing particles. Product concentration was determined by optical density

measurement at 260nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260nm from 0.1 to 1.0, either by direct dilution with PBS or further concentration using Amicon Ultra-0.5 /30K MWCO Centrifugal Filter Device.

Sedimentation velocity AUC data acquisition

Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis was performed using a ProteomeLab™ XL-I (Beckman Coulter). 400 µL sample was loaded into the sample sector of a two sector velocity cell, and 400 µL PBS was loaded into the corresponding reference sector. The sample was placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of 20°C and full vacuum were maintained for one hour. Sedimentation velocity centrifugation was performed at 6,000 RPM, 20°C, 0.003 cm radial step setting, with no delay and with no replicates. Raleigh interference optics were used to simultaneously record radial concentration as a function of time until the smallest sedimenting component cleared the optical window (1.2 hour). Assay throughput was limited to a single sample per run based on absorbance scan collection times of greater than one minute, as well as the large size and rapid sedimentation of Ad2.

AUC data analysis

[0183] The percent full capsid was determined by analyzing approximately 75 scans from interference detection method using the SEDFIT (NIH/see worldwide web at analyticalultracentrifugation.com) continuous size C(S) distribution model. Second (2nd) derivative regularization was applied to the fitting with a confidence level of F statistic/ratio=0.68. The following C(S) parameters were held constant: resolution=250S, S min=10, S max=1500 and frictional ratio=1.86935. RI and TI noise subtractions were applied, and the meniscus position was allowed to float, letting the software choose the optimal position. This model fit the data to the Lamm equation, and the resulting size distribution was a "distribution of sedimentation coefficients" that looked like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) were determined for each component in the distribution. Each AUC run was an independent assay, and each analysis was monitored for the

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following attributes to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\,nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans. The rmsd of this representative example was 0.006584.

Results

[0184] Analytical ultracentrifugation (AUC) using classical boundary sedimentation velocity was used to reveal the particle heterogeneities of recombinant adenovirus serotype 2 vectdor (rAd2) vector preps. To monitor the movement of rAd2 particles in response to a centrifugal force, this mixture of rAd2 capsids was scanned using interference optics along a centrifugal field at defined time intervals. Scans represented the acquisition of concentration data as a function of radius *r*, at times *t*, to yield a series of concentration scans that revealed the complete migration pattern of constituent vector particles in the rAd2 vector prep. Plotting the differential sedimentation coefficient distribution value, C(S), versus the sedimentation coefficient (in Svedberg units, S) yielded distinct peaks with unique sedimentation coefficients rAd2 species (**FIG. 16**). The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.*, 78:1606-19.

[0185] For the rAd2 vector prep shown in **FIG. 16**, 87.8% of the rAd2 vector preparation sedimented with an S value of 731, consistent with a vector preparation consisting predominantly of genome containing capsids. These data confirm that adenoviral particles can be resolved by AUC.

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CLAIMS

What is claimed is:

1. A method of characterizing a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

2. A method to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and

c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes.

3. The method of claim 2 further comprising integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

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4. A method to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

5. A method of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, and

d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

6. A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

7. A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

8. A method of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

9. A method of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method of any one of claims 5-8, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

10. The method of any one of claims 4-9, wherein the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles.

11. The method of any one of claims 4-9, wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes.

12. The method of claim 11, wherein the capsid particles comprising variant sized genomes comprises truncated genomes, aggregates, recombinants and/or DNA impurities.

13. A method of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the

peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation.

14. The method of claim 13, wherein the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes.

15. The method of claim 14, wherein the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities.

16. The method of any one of claims 13-15 further comprising integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles.

17. A method of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the method of claim 16, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

18. The method of any one of claims 1-17, wherein the sedimentation of recombinant viral particles is monitored by absorbance.

19. The method of claim 18, wherein the absorbance is at about 230 nm, 260 nm or 280 nm.

20. The method of claim 18 or 19, wherein the absorbance is at about 260 nm.

21. The method of any one of claims 1-20, wherein the sedimentation of recombinant viral particles is monitored by interference.

22. The method of claim 21, wherein the interference is Rayleigh interference.

23. The method of any one of claims 1-22, wherein the preparation is an aqueous solution.

24. The method of claim 23, wherein the aqueous solution comprises a pharmaceutical formulation.

25. The method of claim 23 or 24, wherein the aqueous solution comprises a buffer.

26. The method of claim 25, wherein the buffer is at physiological pH.

27. The method of claim 25 or 26, wherein the buffer is at physiological osmolality.

28. The method of any one of claims 24-27, wherein the pharmaceutical formulation comprises phosphate buffered saline (PBS).

29. The method of claim 28, wherein the PBS has pH of about 7.2 and an osmolality of about 300 mOsm/L.

30. The method of any one of claims 24-29, wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

31. The method of any one of claims 1-30, wherein the C(S) values are determined by an algorithm that comprises Lamm equation solutions.

32. The method of claim 31, wherein the algorithm is the SEDFIT algorithm.

33. The method of any one of claims 1-32, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge.

34. The method of any one of claims 1-32, wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell.

35. The method of any one of claims 1-33, wherein sedimentation is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell.

36. The method of claim 34, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears an optical window.

37. The method of any one of claims 33-36, wherein at least 30 scans are used to monitor sedimentation of recombinant viral particles.

38. The method of claim 37, wherein about 30 scans are used to monitor sedimentation of recombinant viral particles.

39. The method of claim 37, wherein about 30 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

40. The method of claim 37, wherein about 30 to about 50 scans are used to monitor sedimentation of recombinant viral particles.

41. The method of claim 37, wherein about 50 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

42. The method of claim any one of claims 31-41, wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68.

43. The method of claim 42, wherein the regularization is a second derivative regularization.

44. The method of claim 42, wherein the regularization is Max entropy regularization.

45. The method of any one of claims 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.90.

46. The method of any one of claims 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.99.

47. The method of any one of claim 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68.

48. The method of any one of claims 31-47, wherein the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software.

49. The method of claim 48, wherein resolution is about 200S to about 1000S.

50. The method of claim 48 or 49, wherein resolution is about 200S.

51. The method of any one of claims 48-50, wherein S min is about 1.

52. The method of any one of claims 48-51, wherein Smax is about 100S to about 1000S.

53. The method of any one of claims 48-51, wherein Smax is about 200S to about 5000S.

54. The method of any one of claims 48-51, wherein Smax is about 200S.

55. The method of any one of claims 48-54, wherein the frictional ratio is left to float to a value determined by centrifugation software.

56. The method of claim any one of 48-54, wherein the frictional ratio is about 1.0.

57. The method of any one of claims 31-56, wherein radial invariant (RI) and time invariant (TI) noise subtractions are applied.

58. The method of any one of claims 1-57, wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds.

59. The method of claim 58, wherein the sedimentation of recombinant viral particles is monitored about every 10 seconds.

60. The method of claim 58, wherein the sedimentation of recombinant viral particles is monitored about every 60 seconds

61. The method of any one of claims 1-60, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm.

62. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 10,000 rpm.

63. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 10,000 rpm to about 20,000 rpm.

64. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 15,000 rpm to about 20,000 rpm.

65. The method of any one of claims 1-64, wherein the boundary sedimentation velocity is performed at about 4°C to about 20 °C.

66. The method of claim 65, wherein the boundary sedimentation velocity is performed at about 4°C.

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67. The method of any one of claims 1-66, wherein the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle.

68. A method of evaluating a process for the production of recombinant viral particles comprising the method of any one of claims 1 to 67, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles.

69. The method of claim 68, wherein the viral particles are rAAV particles.

70. The method of claim 69, wherein the rAAV particles are produced from a producer cell line.

71. The method of claim 69, wherein the rAAV particles are produced by triple transfection of i) nucleic acid encoding AAV rep and cap, ii) rAAV vector sequences, and iii) nucleic acid encoding adenovirus helper functions.

72. The method of claim 69, wherein the recombinant viral particles are produced by an AAV/HSV hybrid

73. The method of claim 69, wherein the recombinant viral particles are produced from a insect cell.

74. The method of claim 69, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell.

75. The method of claim 69, wherein the recombinant viral particles are produced by introduction of one or more nucleic acids encoding AAV vector sequences, AAV rep and cap

coding regions, and AAV helper virus functions to a suitable host cell, wherein the one or more nucleic acids are introduced to the cell using a recombinant helper virus.

76. The method of claim 75, wherein the recombinant helper virus is an adenovirus, a herpes simplex virus or a baculovirus.

77. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding adenovirus vector sequences and adenovirus replication and packaging sequences to a suitable host cell.

78. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding lentivirus vector sequences and/or lentivirus replication and packaging sequences to a suitable host cell.

79. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding HSV vector sequences and/or HSV replication and packaging sequences to a suitable host cell.

80. A method for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising

a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant viral particles;

c) isolating the recombinant viral particles produced by the host cell; and

d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the methods of any one of claims 1 to 79.

81. The method of claim 80, wherein the p5 promoter is located 3' to the rep and/or cap coding region.

82. The method of claim 80 or 81, wherein the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

83. The method of any one of the preceding claims wherein the recombinant viral particles have been purified using one or more purification steps.

84. The method of claim 67, 69-76, 80-83 wherein the recombinant viral particles comprise a self-complementary AAV (scAAV) genome.

85. The method of claim 84, wherein the method is used to detect the presence of recombinant viral particles comprising the monomeric form of a scAAV genome or the dimeric form of a scAAV genome.

86. The method of any one of claims 67, 69-76, 80-85,wherein the recombinant viral particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1).

87. The method of any one of claims 67, 69-76, 80-86, wherein the recombinant viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, an AAV12 ITR, an AAV DJ ITR, a goat AAV ITR, a bovine AAV ITR, or a mouse AAV ITR.

88. The method of claim 86 or 87, wherein the AAV capsid comprises a tyrosine mutation or a heparin binding mutation,

89. The method of claim 67 or 68, where the recombinant viral particle is a recombinant adenoviral particle.

90. The method of claim 89, wherein the recombinant adenoviral particle comprises an capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, or porcine Ad type 3.

91. The method of claim 90, wherein the recombinant adenoviral particle comprises a variant of an adenovirus serotype 2 capsid or a variant of an adenoviral serotype 5 capsid.

92. The method of claim 67 or 68, where the recombinant viral particle is a recombinant lentiviral particle.

93. The method of claim 92, wherein the recombinant lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114 or variants therein.

94. The method of claim 67 or 68, where the recombinant viral particle is a rHSV particle.

95. The method of claim 94, wherein the HSV particle is an HSV-1 particle or an HSV-2 particle.

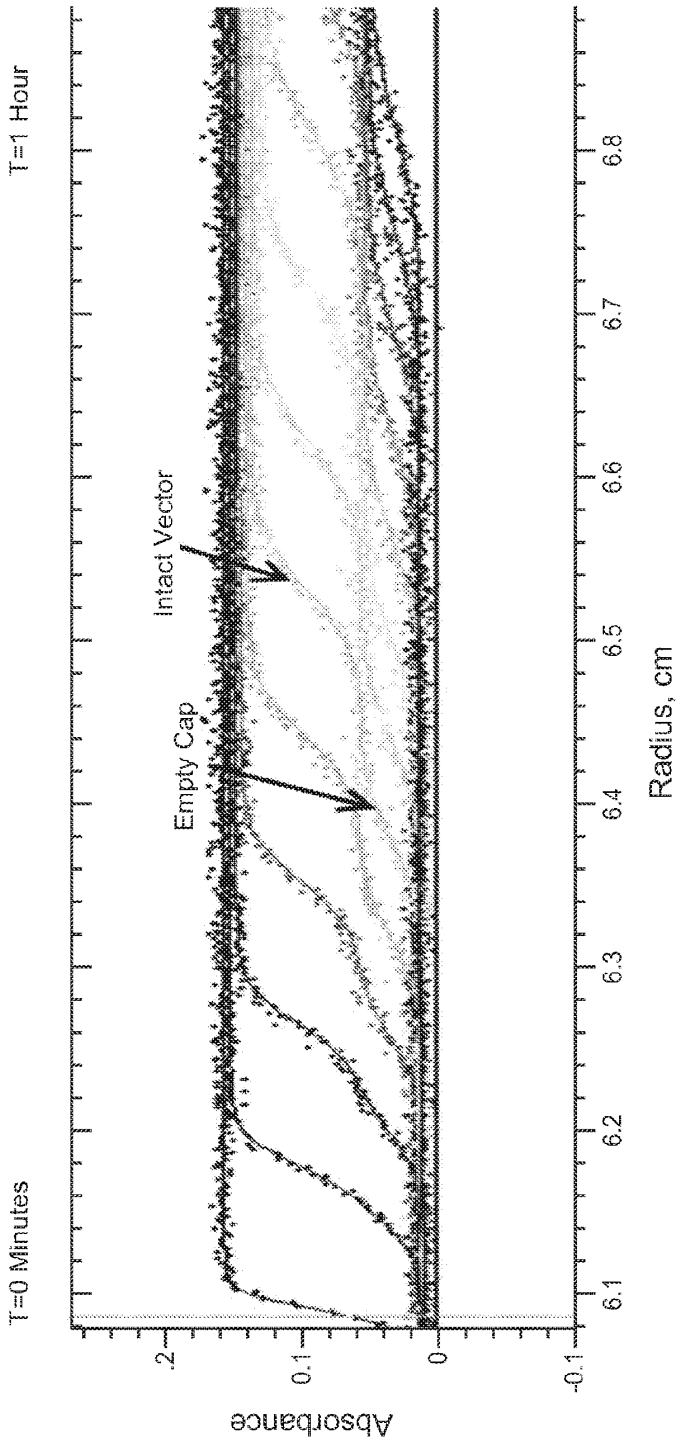

T=1 Hour
T=0 Minutes
Intact Vector
Empty Cap
Absorbance
.2
0.1
0
-0.1
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
Radius, cm

FIG. 1A

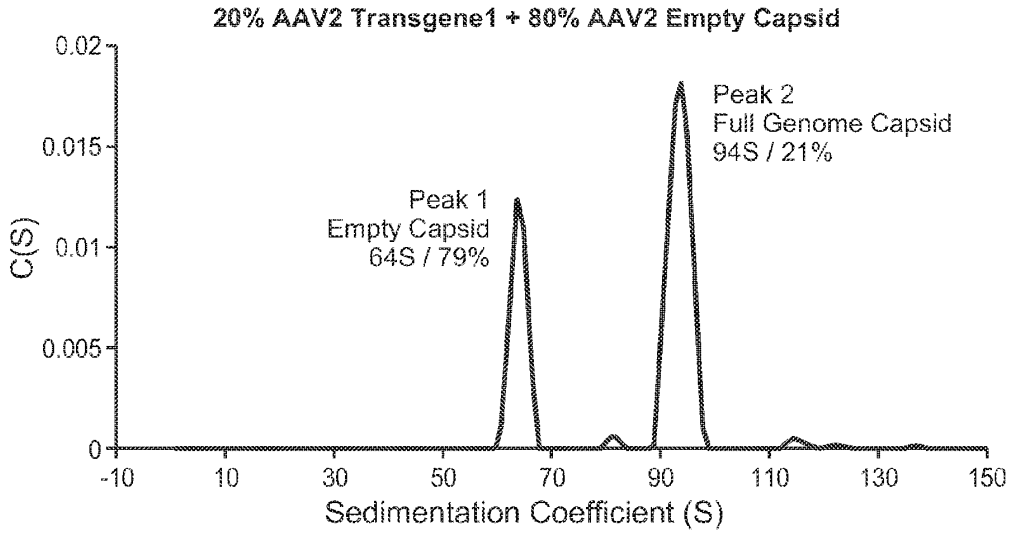


FIG. 1B

Novartis Exhibit 1002, Page 93 of 591

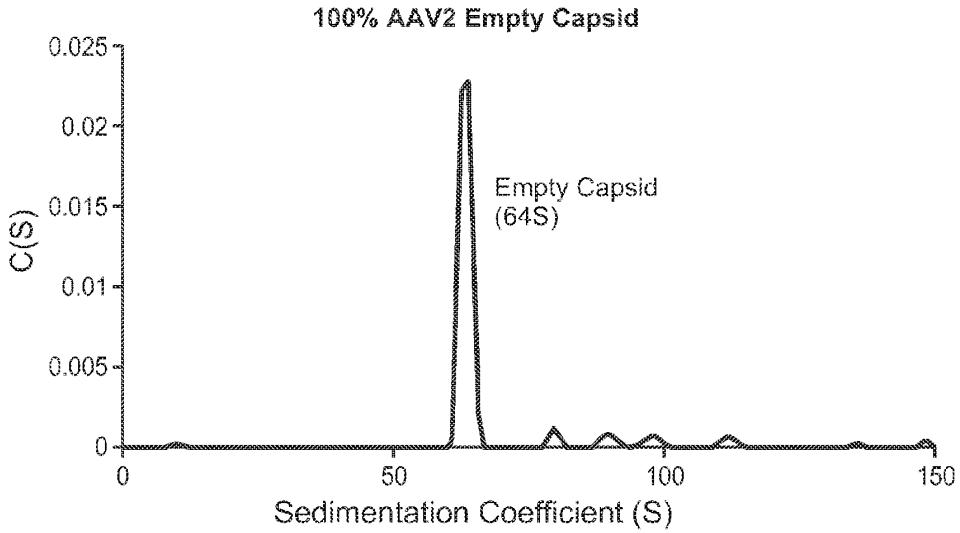

100% AAV2 Empty Capsid
0.025
0.02
0.015
0.01
0.005
0
C(S)
Empty Capsid
(64S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2A

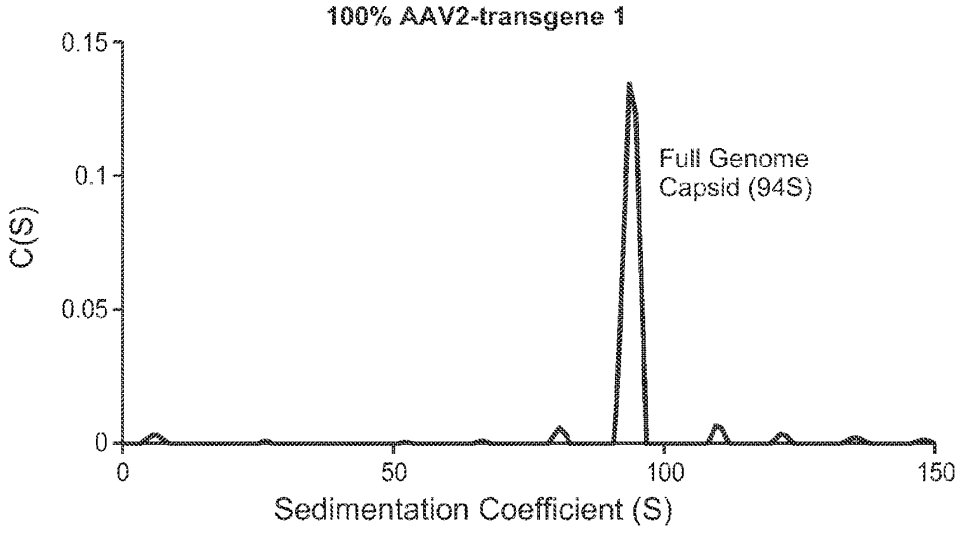

100% AAV2-transgene 1
0.15
0.1
0.05
0
C(S)
Full Genome
Capsid (94S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2B

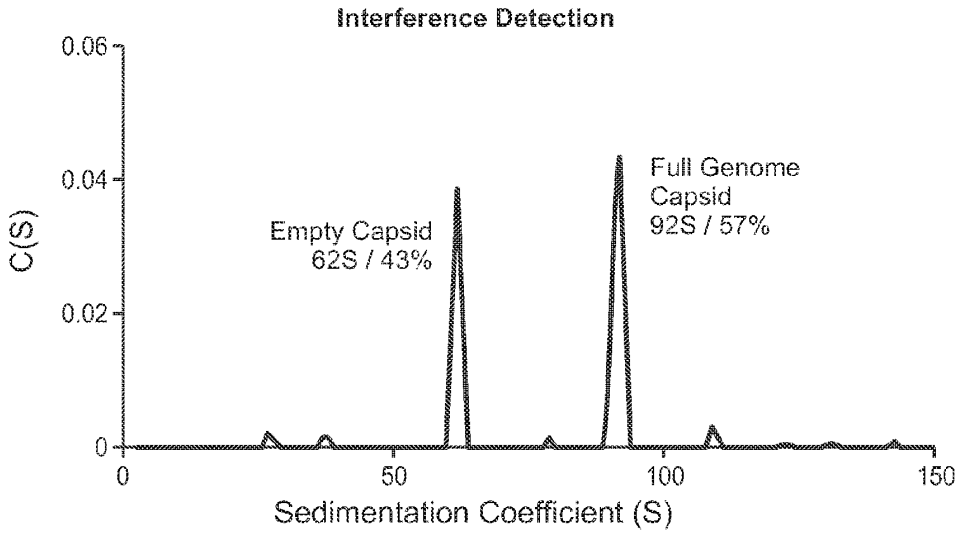

Interference Detection
0.06
0.04
0.02
0
C(S)
Empty Capsid
62S / 43%
Full Genome
Capsid
92S / 57%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3A

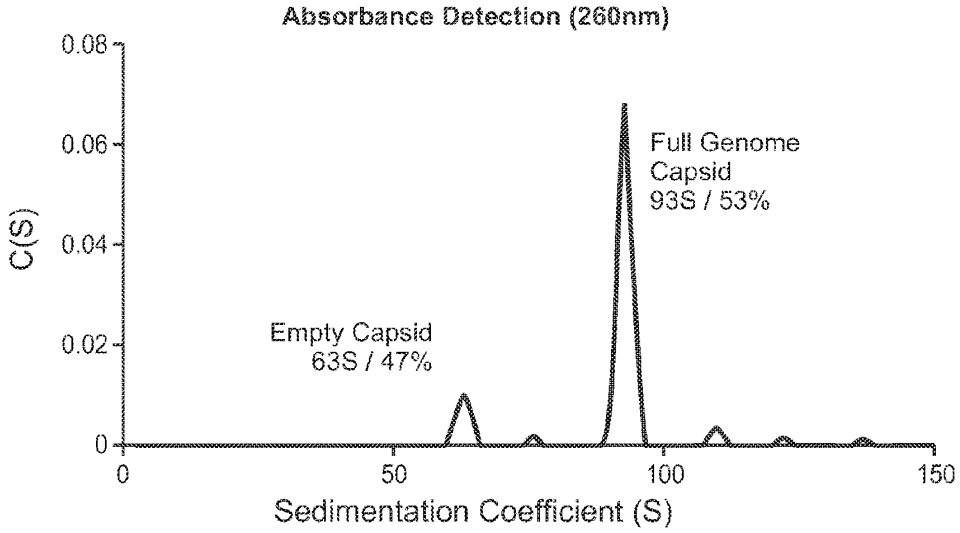

Absorbance Detection (260nm)
0.08
0.06
0.04
0.02
0
C(S)
Empty Capsid
63S / 47%
Full Genome
Capsid
93S / 53%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3B

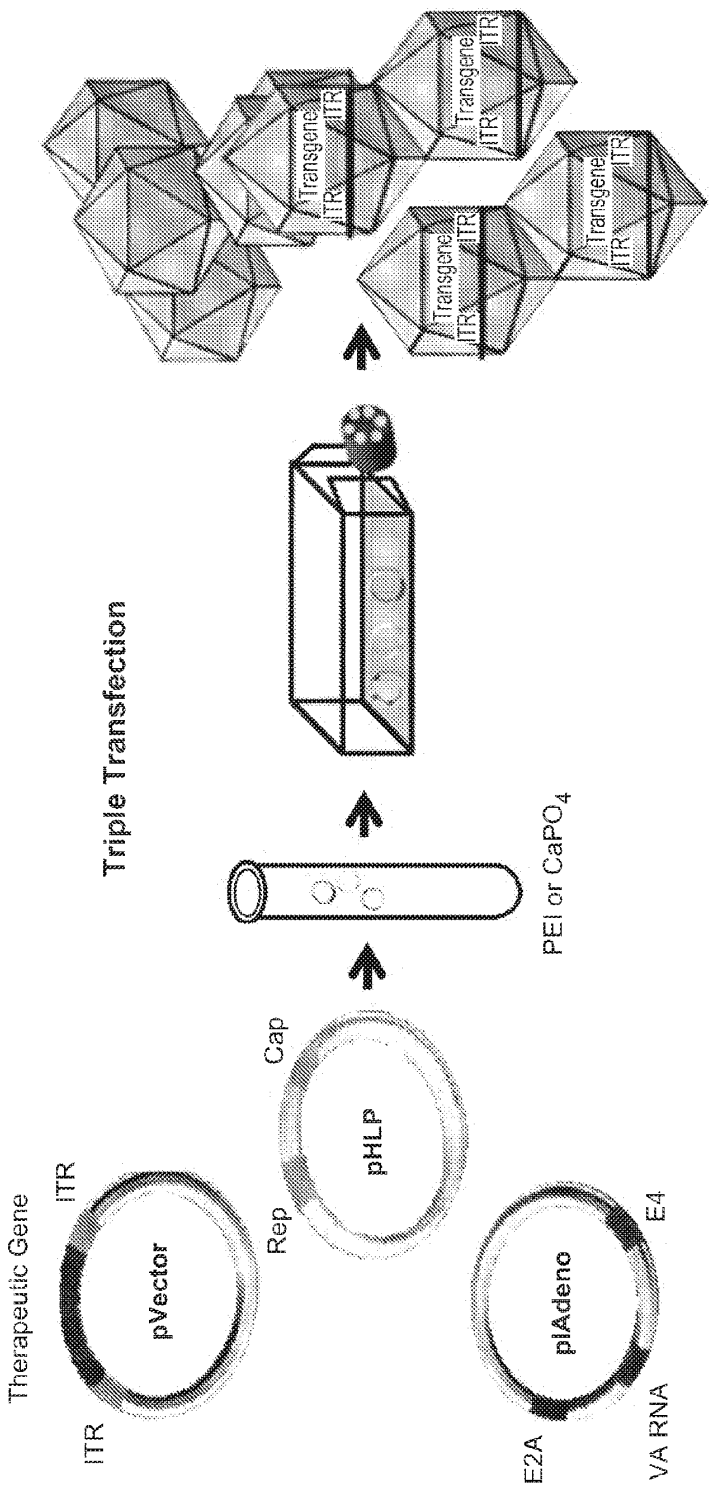


FIG. 4

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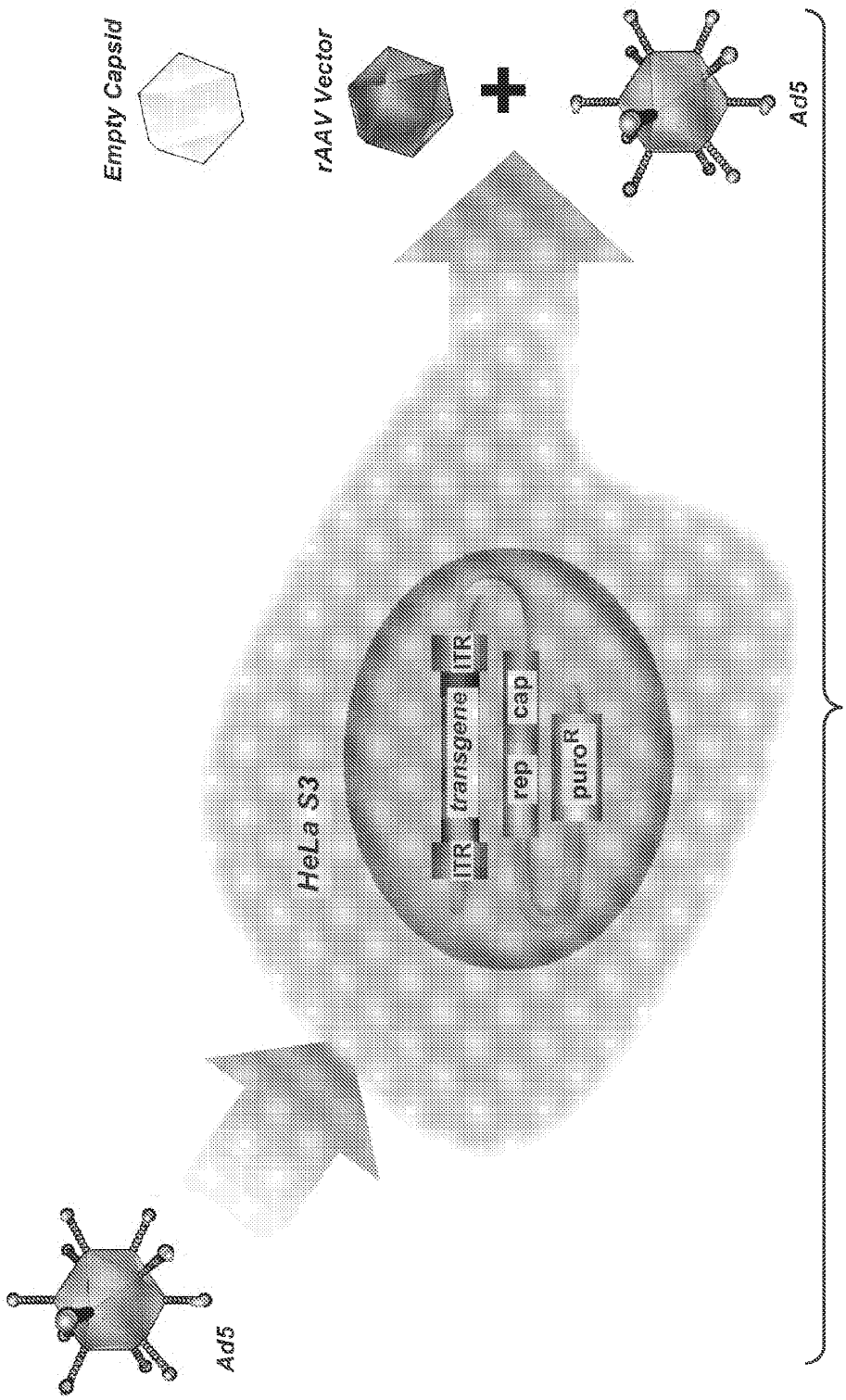
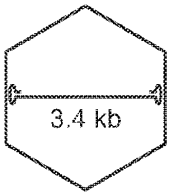

Empty Capsid
rAAV Vector
Ad5
HeLa S3
ITR
transgene
ITR
rep
cap
puroR
Ad5

FIG. 5

FIG. 6A



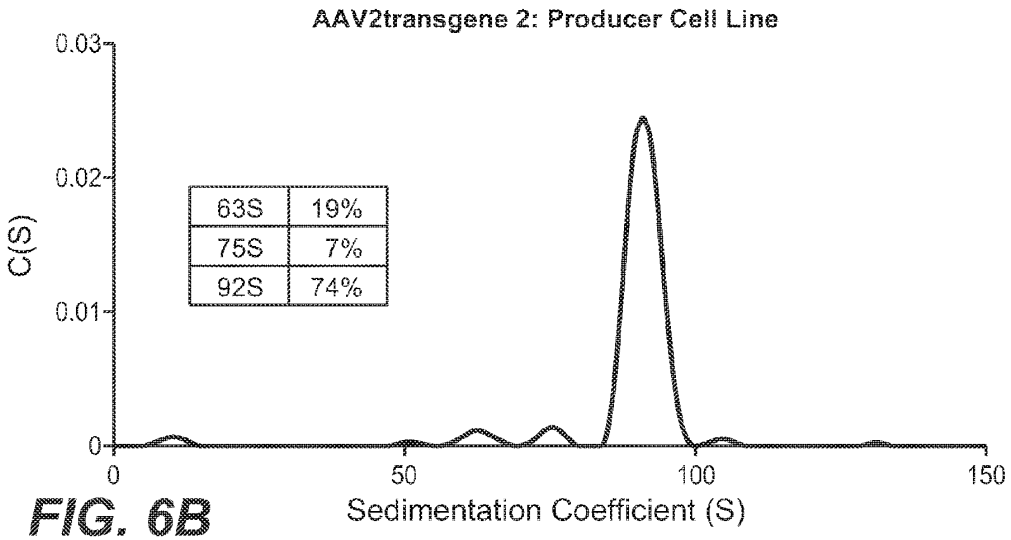


FIG. 6B

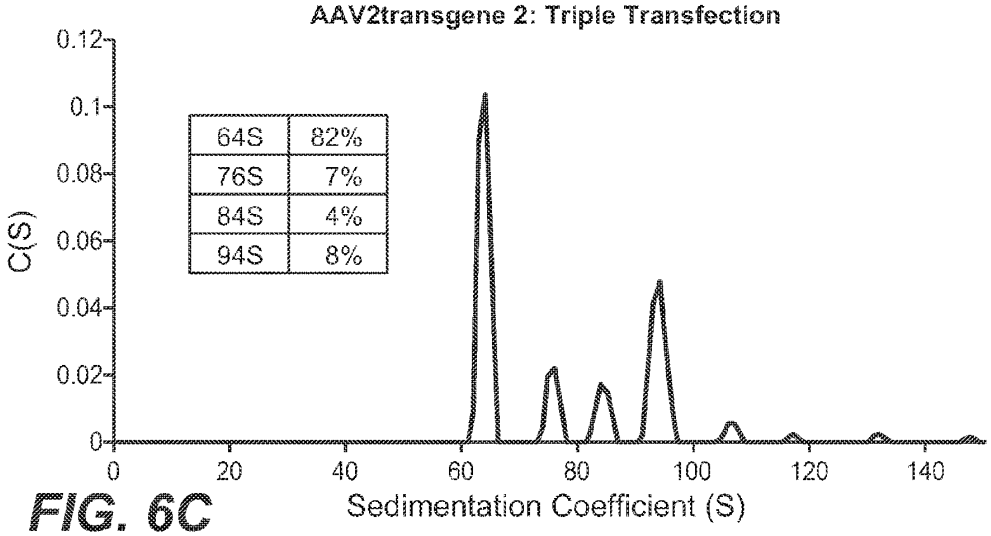


FIG. 6C

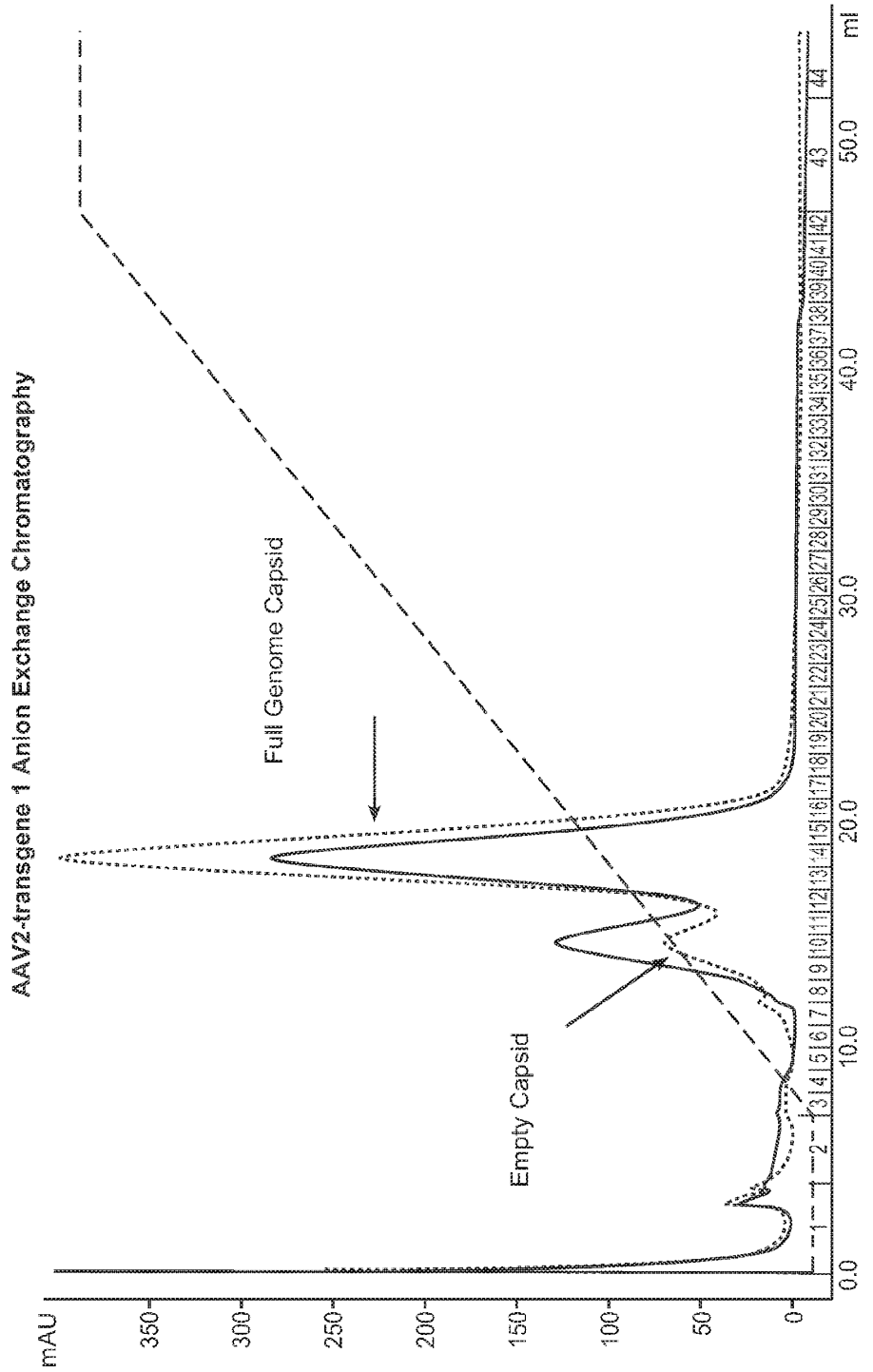

AAV2-transgene 1 Anion Exchange Chromatography
Full Genome Capsid
Empty Capsid
mAU
350
300
250
200
150
100
50
0
0.0
10.0
20.0
30.0
40.0
50.0
ml

FIG. 7A

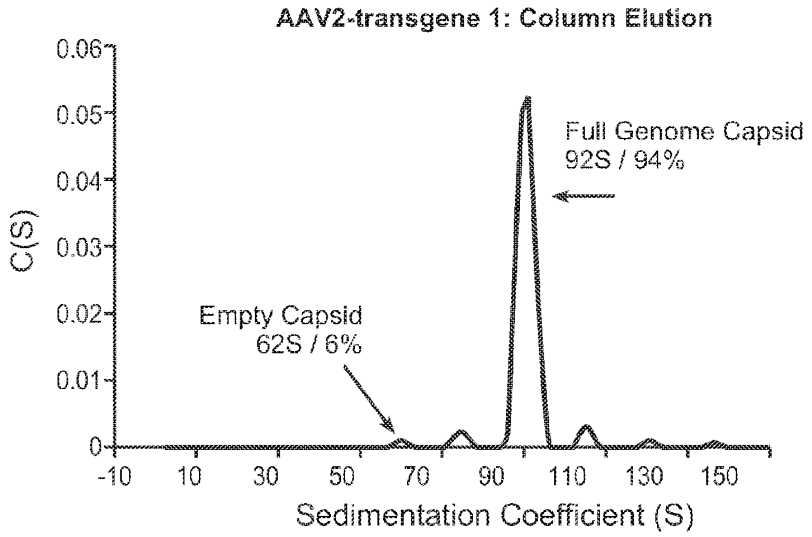

AAV2-transgene 1: Column Elution
0.06
0.05
0.04
0.03
0.02
0.01
0
C(S)
Full Genome Capsid
92S / 94%
Empty Capsid
62S / 6%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7B

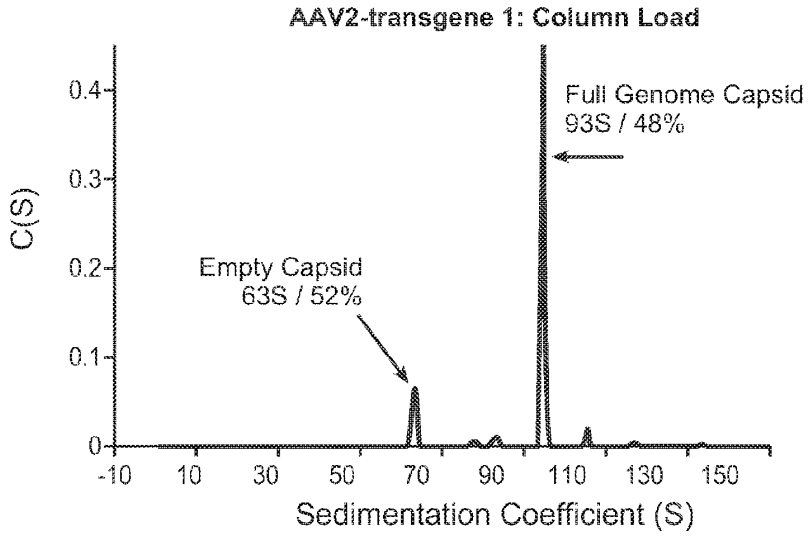

AAV2-transgene 1: Column Load
0.4
0.3
0.2
0.1
0
C(S)
Full Genome Capsid
93S / 48%
Empty Capsid
63S / 52%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7C

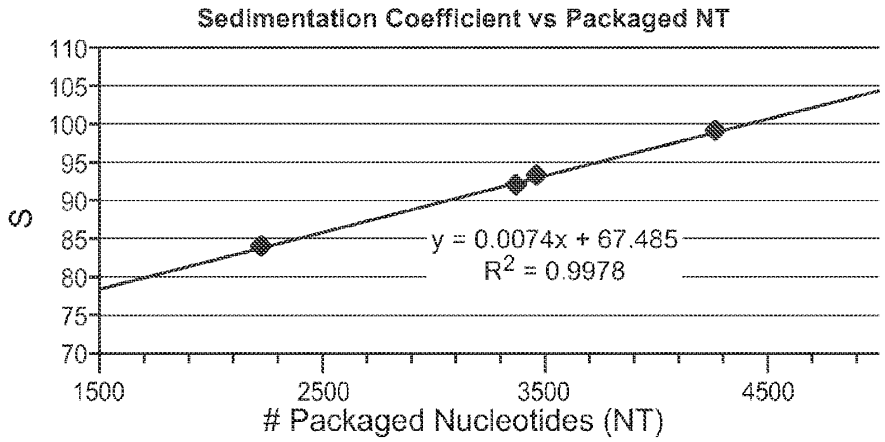

Sedimentation Coefficient vs Packaged NT
S
110
105
100
95
90
85
80
75
70
y = 0.0074x + 67.485
R2 = 0.9978
1500
2500
3500
4500
Packaged Nucleotides (NT)

FIG. 8

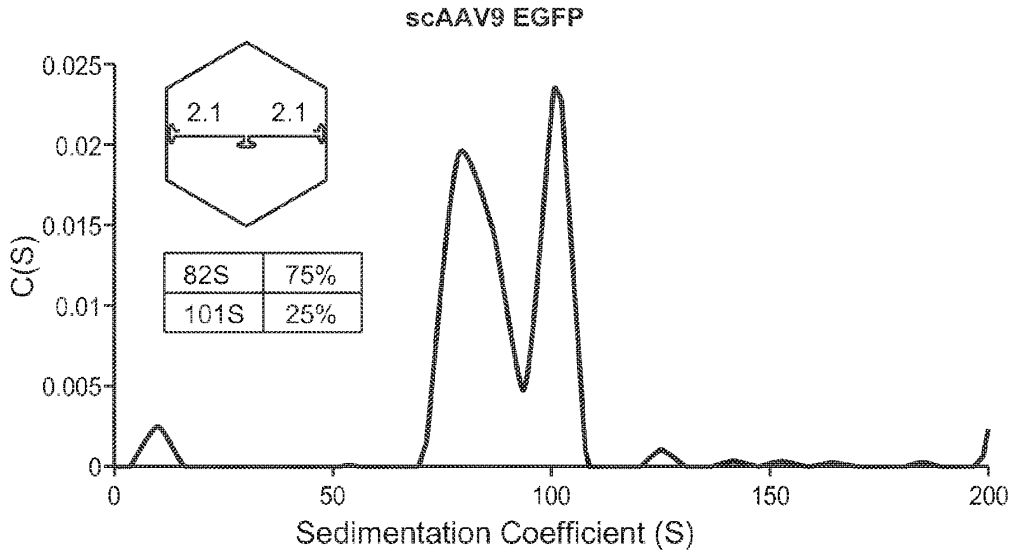

scAAV9 EGFP
C(S)
0.025
0.02
0.015
0.01
0.005
0
2.1
2.1
82S 75%
101S 25%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9A

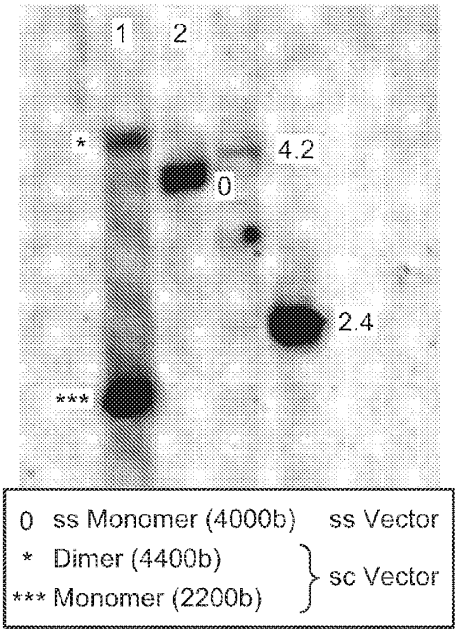


FIG. 9B

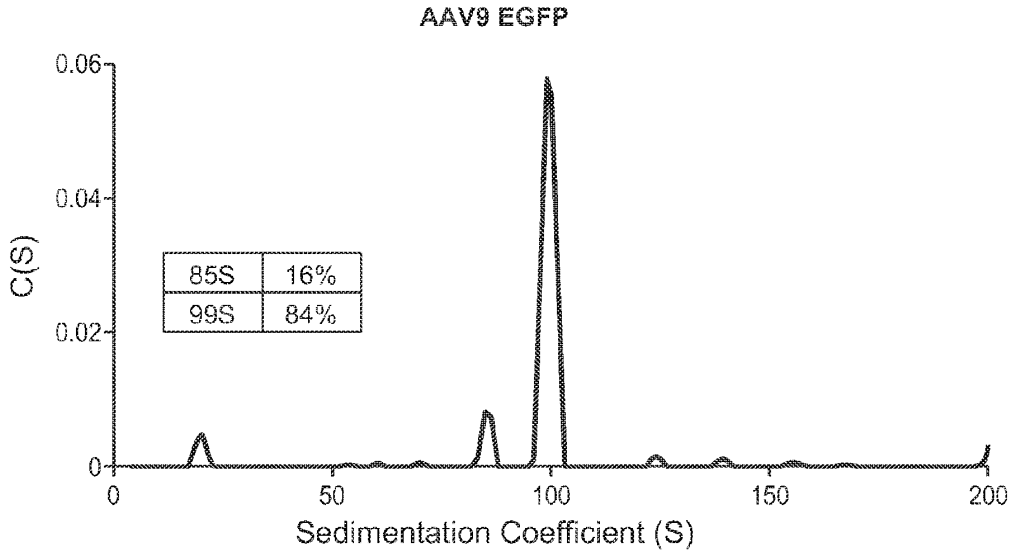


FIG. 9C

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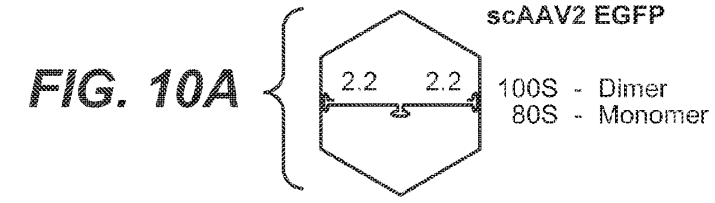


FIG. 10A

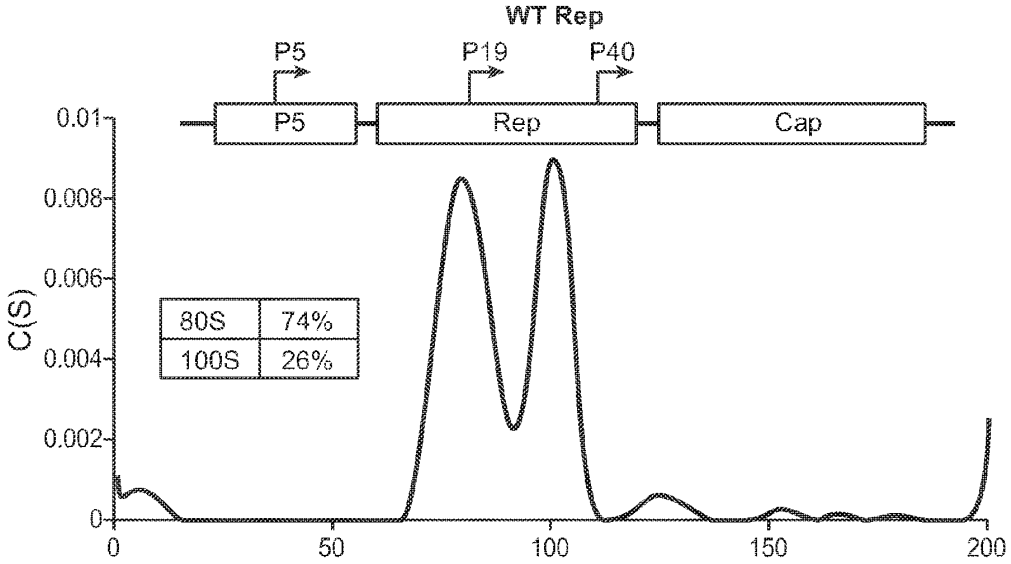


FIG. 10B

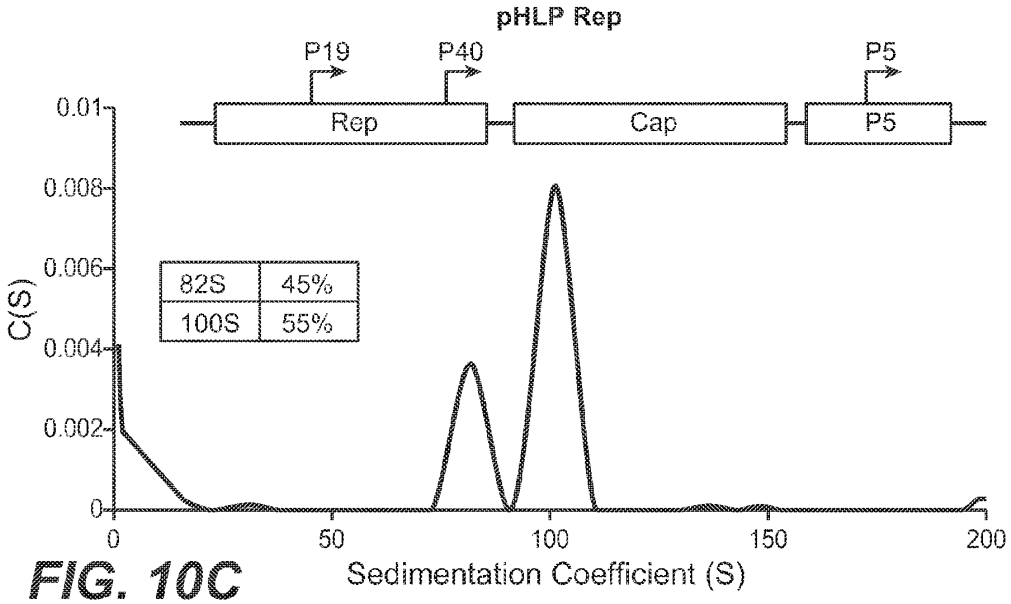


FIG. 10C

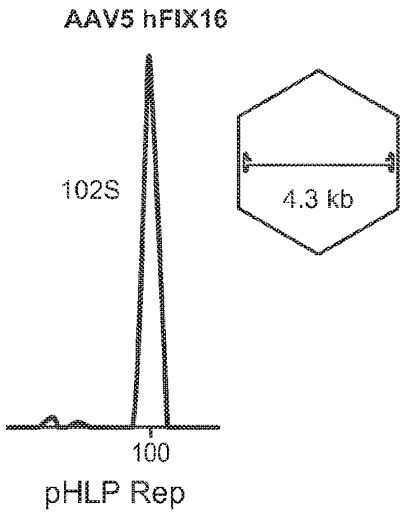


FIG. 11A

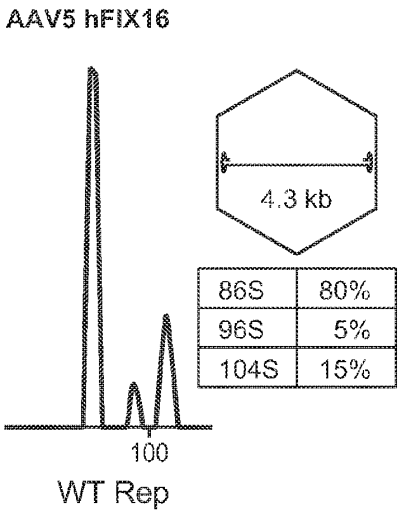


FIG. 11B

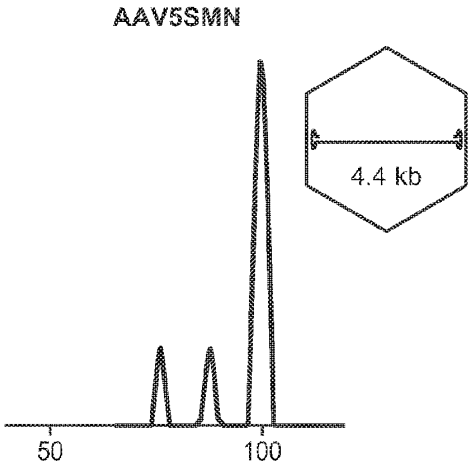


FIG. 11C

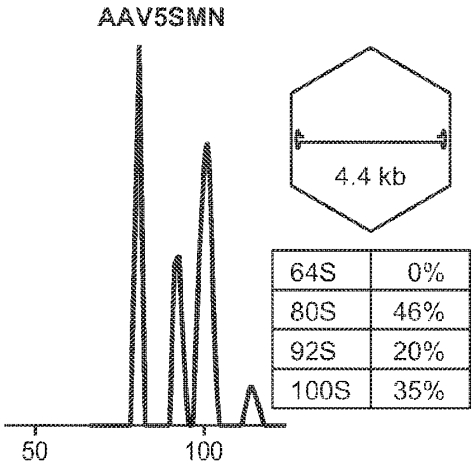
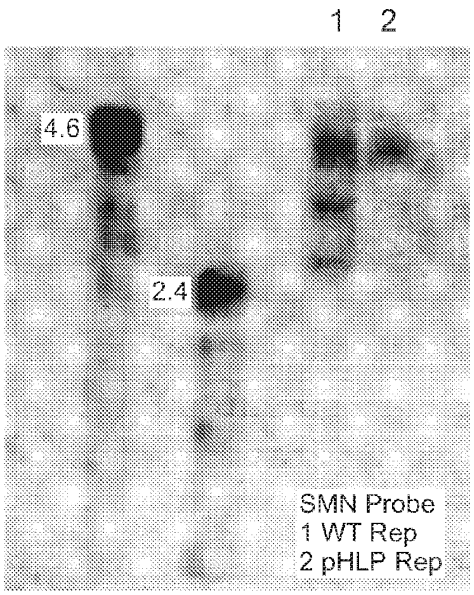
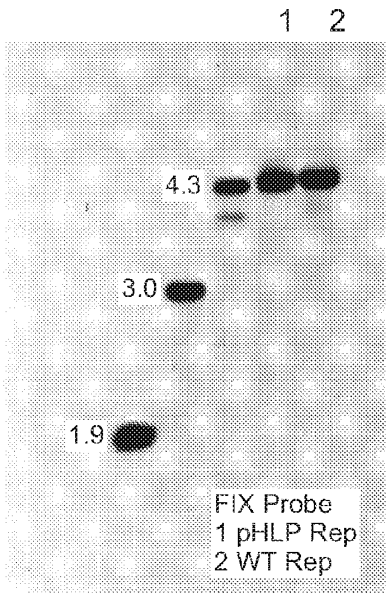


FIG. 11D



SMN Probe

FIG. 12A



FIX Probe

FIG. 12B

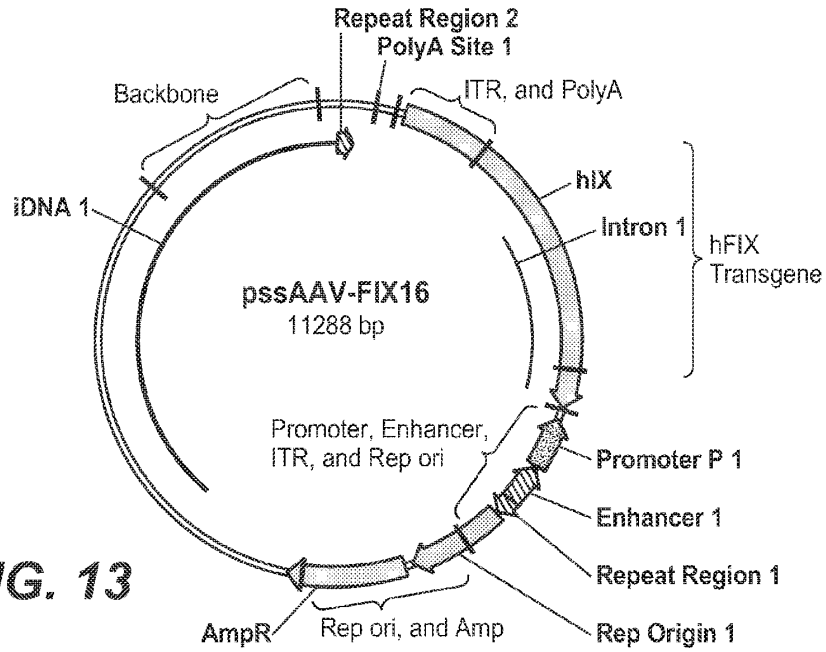
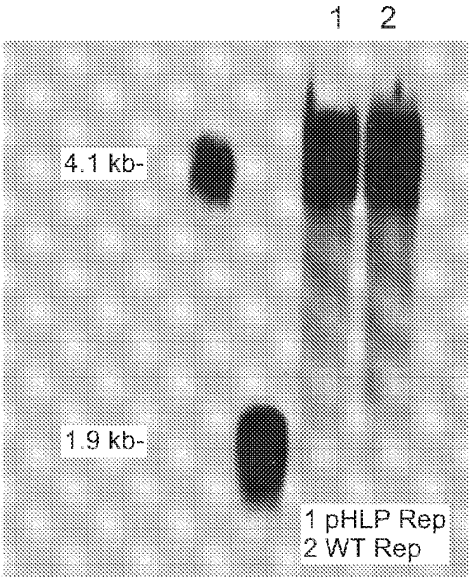
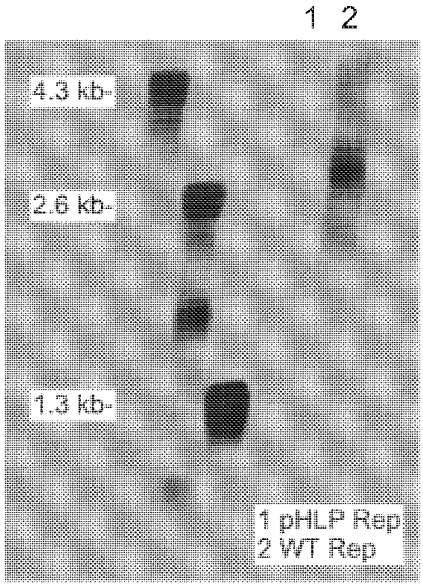


FIG. 13



Transgene

FIG. 14A



Rep ori & Amp

FIG. 14B

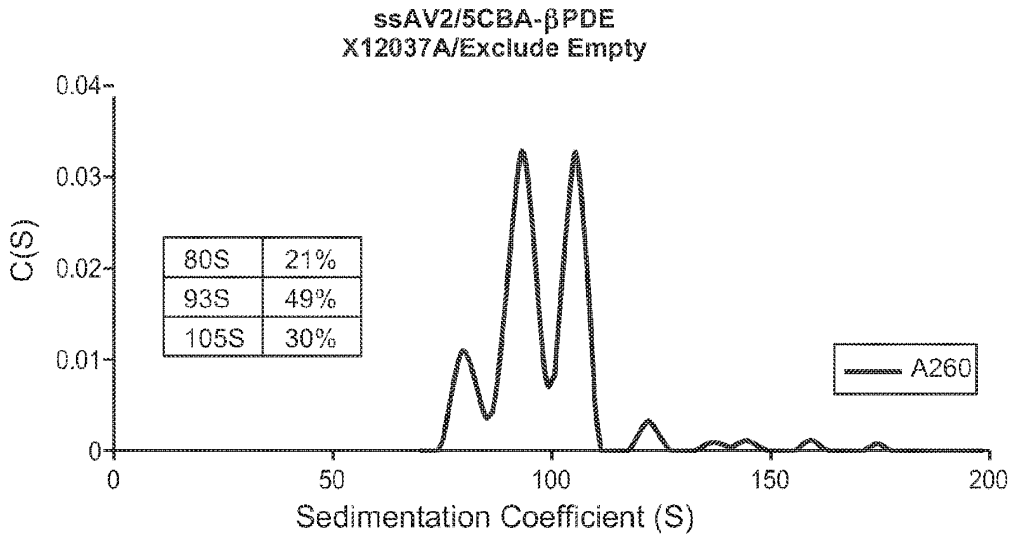


FIG. 15A

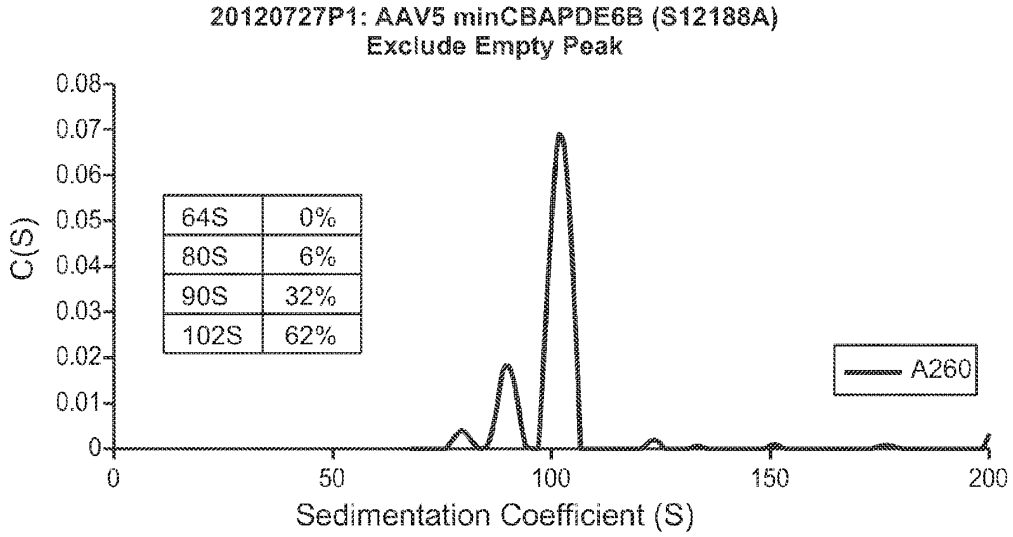


FIG. 15B

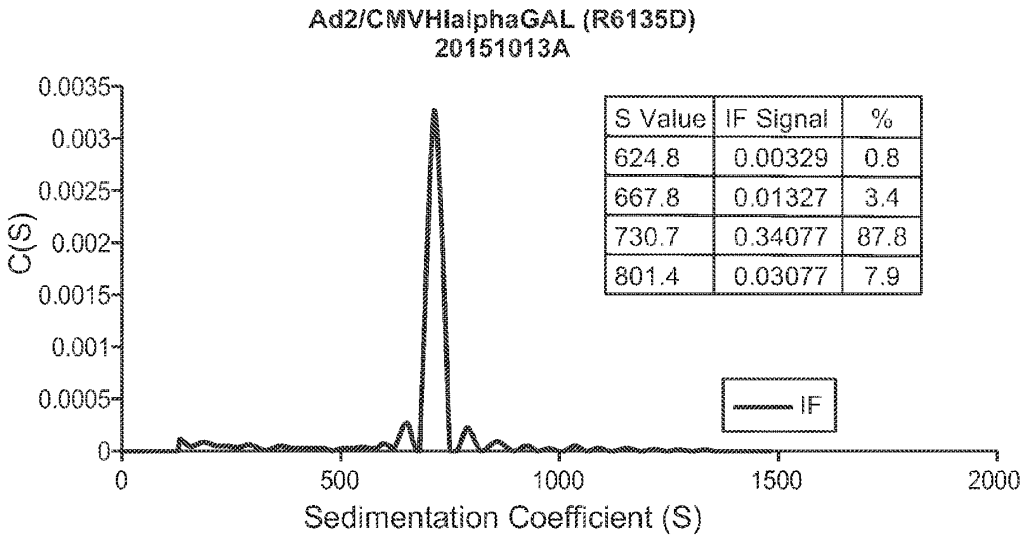

Ad2/CMVHIalphaGAL (R6135D)
20151013A
C(S)
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
500
1000
1500
2000
Sedimentation Coefficient (S)
IF
S Value | IF Signal | %
624.8 | 0.00329 | 0.8
667.8 | 0.01327 | 3.4
730.7 | 0.34077 | 87.8
801.4 | 0.03077 | 7.9

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/013947

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/02 G01N9/30
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Ewoud Van Tricht ET AL: "MSc Chemistry Analytical Sciences Virus Particle Characterization Techniques to Quantify Virus Particle Aggregation and Integrity", , 1 July 2013 (2013-07-01), pages 1-51, XP055258342, Retrieved from the Internet: URL:https://esc.fnwi.uva.nl/thesis/centraal/files/f318319369.pdf [retrieved on 2016-03-15] Whole doc., in particular p.23-24 (esp. Fig.7); p.44 (bottom para.)-45; Table 2 (p.46); Table 3 (p.48). ----- -/--	1-95

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2016	12/04/2016

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

2

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

Novartis Exhibit 1002, Page 109 of 591

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERKOWITZ ET AL: "Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 362, no. 1, 1 March 2007 (2007-03-01) , pages 16-37, XP022056699, ISSN: 0003-2697, DOI: 10.1016/J.AB.2006.11.031 Whole doc., in partic. abstract; p.17; p.18(col.2, l. 19-21); Fig.8C; p.31, col.1	1-95
X	XIAOYU YANG ET AL: "Determination of Particle Heterogeneity and Stability of Recombinant Adenovirus by Analytical Ultracentrifugation in CsCl Gradients", JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 97, no. 2, 1 February 2008 (2008-02-01), pages 746-763, XP055258325, WASHINGTON, US ISSN: 0022-3549, DOI: 10.1002/jps.21008 Whole doc., in partic. abstract; p.749, col.2; p.751, col.2; p.753	1-95
A	J. FRASER WRIGHT: "Product-Related Impurities in Clinical-Grade Recombinant AAV Vectors: Characterization and Risk Assessment", BIOMEDICINES, vol. 2, no. 1, 3 March 2014 (2014-03-03), pages 80-97, XP009183697, Whole doc., in partic. abstract; table 2 (p.85)	1-95
X,P	BRENDA BURNHAM ET AL: "Analytical Ultracentrifugation as an Approach to Characterize Recombinant Adeno-Associated Viral Vectors", HUMAN GENE THERAPY METHODS, vol. 26, no. 6, 1 December 2015 (2015-12-01), pages 228-242, XP055258343, ISSN: 1946-6536, DOI: 10.1089/hgtb.2015.048 Whole doc. - applicants scientific disclosure of invention.	1-95

2

I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: July 18, 2017
Electronic Signature for Ruth Lang: /Ruth Lang/

Docket No.: 159792010300
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Catherine R. O'RIORDAN et al.

Application No.: Not Yet Assigned
Int'l Application No. PCT/US2016/013947

Confirmation No.: Not Yet Assigned

Filed: Concurrently Herewith
Int'l Filing Date: January 19, 2016

Art Unit: Not Yet Assigned

For: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Examiner: Not Yet Assigned

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

MS PCT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, Applicant respectfully requests entry of this Preliminary Amendment for the above-captioned patent application.

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

Remarks/Arguments begin on page 3 of this paper.

pa-1793293

AMENDMENTS TO THE SPECIFICATION

Please amend paragraph **[0001]**, as follows:

[0001] This application is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2016/013947 filed January 19, 2016, which claims priority to U.S. Provisional Application No. 62/105,714, filed [[on]] January 20, 2015, ~~the content~~ each of which is ~~hereby~~ incorporated herein by reference in its entirety ~~for all purposes~~.

REMARKS

By virtue of this amendment, paragraph **[0001]** is amended to update the cross reference to related applications information. No new matter is added.

Entry of the amendments to the specification is respectfully requested.

pa-1793293

CONCLUSION

If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below

Dated: July 18, 2017

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
Brian A. Donahue
Registration No.: 58,206
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, California 94304-1018
(650) 813-5632

pa-1793293

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)

The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Notes Nos. VIII, VIII(i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

Declaration of Inventorship (Rules 4.17(iv) and 51*bis*.1(a)(iv))
for the purposes of the designation of the United States of America:

I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/ US2016/013947 (if furnishing declaration pursuant to Rule 26*ter*.)

I hereby declare that the above identified international application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Name: Catherine R. O'RIORDAN

Residence: Weban, Massachusetts
(city and either US state, if applicable, or country)

Mailing Address: c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey, 08807: UNITED STATES OF AMERICA

Catherine O'Riordan — 3/14/2016
Inventor's Signature — Date
(The signature must be that of the inventor, not that of the agent)

Name: Brenda BURNHAM

Residence: Hopkinton, MA
(city and either US state, if applicable, or country)

Mailing Address: c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807: UNITED STATES OF AMERICA

Inventor's Signature — Date
(The signature must be that of the inventor, not that of the agent)

Name:

Residence:
(city and either US state, if applicable, or country)

Mailing Address:

Inventor's Signature — Date
(The signature must be that of the inventor, not that of the agent)

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)

The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Boxes Nos. VIII, VIII(i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

Declaration of inventorship (Rules 4.17(iv) and 51*bis*.1(a)(iv)) for the purposes of the designation of the United States of America:

I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/ US2016/013947 (if furnishing declaration pursuant to Rule 26*ter*.)

I hereby declare that the above identified international application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Name: Catherine R. O'RIORDAN

Residence: Waban, Massachusetts
(city and either US state, if applicable, or country)

Mailing Address: c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807; UNITED STATES OF AMERICA

Inventor's Signature ____ Date ____
(The signature must be that of the inventor, not that of the agent)

Name: Brenda BURNHAM

Residence: Hopkinton, MA
(city and either US state, if applicable, or country)

Mailing Address: c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807; UNITED STATES OF AMERICA

Brenda Burnham 3/16/2016
Inventor's Signature Date
(The signature must be that of the inventor, not that of the agent)

Name:

Residence:
(city and either US state, if applicable, or country)

Mailing Address:

Inventor's Signature ____ Date ____
(The signature must be that of the inventor, not that of the agent)

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 89300

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter to:

[X] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

GENZYME CORPORATION

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	/Andrea Q. Ryan/	Date (Optional)	July 23, 2014
Name	Andrea Q. Ryan		
Title	Attorney-In-Fact		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[X] Total of 1 forms are submitted.

sf-3407816

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159792010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Catherine	R.	O'RIORDAN	

Residence Information (Select One) ● US Residency Non US Residency Active US Military Service

City	Bridgewater	State/Province	NJ	Country of Residence	US

Mailing Address of Inventor:

Address 1	c/o Sanofi
Address 2	55 Corporate Drive
City	Bridgewater
State/Province	NJ
Postal Code	08807
Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Brenda		BURNHAM	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

City	Hopkinton	State/Province	MA	Country of Residence	US

Mailing Address of Inventor:

Address 1	c/o Sanofi
Address 2	55 Corporate Drive
City	Bridgewater
State/Province	NJ
Postal Code	08807
Country	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159792010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	89300		
Email Address	patentdocketa@mofo.com	Add Email	Remove Email

Application Information:

Title of the Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES		
Attorney Docket Number	159792010300	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	17	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	89300		

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159792010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	a 371 of international	PCT/US2016/013947	2016-01-19
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2016/013947	Claims benefit of provisional	62105714	2015-01-20

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

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

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159792010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Authorization or Opt-Out of Authorization to Permit Access:

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

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

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

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

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

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

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

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

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

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

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

PTO/AIA/14 (11-15)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159792010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 — Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Genzyme Corporation		
Mailing Address Information For Applicant:			
Address 1	500 Kendall Street		
Address 2			
City	Cambridge	**State/Province**	MA
Country	US	Postal Code	02142
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159792010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Brian A. Donahue/			Date (YYYY-MM-DD)	2017-07-18
First Name	Brian A.	Last Name	Donahue	Registration Number	58206

Additional Signature may be generated within this form by selecting the Add button. Add

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017. OMB 0651-0032
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 contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	159792010300
		Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES		

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Filer:	Brian Arthur Donahue/Ruth Lang
Attorney Docket Number:	159792010300

Filed as Large Entity

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
NATIONAL STAGE FEE	1631	1	280	280
NATL STAGE SEARCH FEE - REPORT PROVIDED	1642	1	480	480
NATIONAL STAGE EXAM - ALL OTHER CASES	1633	1	720	720
Pages:				
NATL STAGE APPL SZ FEE PER 50 PGS >100	1681	1	400	400
Claims:				
Miscellaneous-Filing:				
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		1880

Electronic Acknowledgement Receipt	
EFS ID:	29818871
Application Number:	15544498
International Application Number:	PCT/US2016/013947
Confirmation Number:	2986
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Ruth Lang
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	159792010300
Receipt Date:	18-JUL-2017
Filing Date:	
Time Stamp:	18:39:03
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1880
RAM confirmation Number	071917INTEFSW00005309031952
Deposit Account	031952
Authorized User	Ruth Lang
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.17 (Patent application and reexamination processing fees) 37 CFR 1.19 (Document supply fees)	

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

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

37 CFR 1.492(a) (Basic national fee only)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal of New Application	15979_20103_00_Transmittal.pdf	47921 9a392e48824bdf1d8ab1b8cb5f539eae14082da2	no	3
Warnings:					
Information:					
2	Documents submitted with 371 Applications	15979_20103_00_PCTPublishedApplication.pdf	12790610 00bcad6e45554f3d7994ccced00dc6c6c47aa913	no	107
Warnings:					
Information:					
3		15979_20103_00_Prelim.pdf	26839 0c77f530fc0ee02e24a1e3d4d14b466075c63006	yes	4

Multipart Description/PDF files in .zip description

Document Description	Start	End
Preliminary Amendment	1	1
Specification	2	2
Applicant Arguments/Remarks Made in an Amendment	3	4

Warnings:					
Information:					
4	Oath or Declaration filed	15979_20103_00_Declarations.pdf	247956 3baf92b74f86fa0d36b3230d349cd71f2faa8301	no	2
Warnings:					
Information:					
5	Power of Attorney	15979_20103_00_POA.pdf	121830 2797ed3068e618dfc7d6eb115292f97a9f97cc55	no	1

Warnings:					
Information:					
6	Application Data Sheet	15979_20103_00_ADS.PDF	1822705 e8914c26166c80b0b45ea432fb7699eedf3fb1b4	no	8
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	36989 0fe478d827de7ed6d13a48ad6544153d68daa674	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	15094850		

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.

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)

The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Boxes Nos. VIII, VIII(i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

Declaration of inventorship (Rules 4.17(iv) and 51*bis*.1(a)(iv)) for the purposes of the designation of the United States of America:

I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/ _____ (if furnishing declaration pursuant to Rule 26*ter*.)

I hereby declare that the above identified international application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Name: Catherine R. O'RIORDAN

Residence: Waban, Massachuetts
(city and either US state, if applicable, or country)

Mailing Address: c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807: UNITED STATES OF AMERICA

Inventor's Signature _____ Date _____
(The signature must be that of the inventor, not that of the agent)

Name: Brenda BURNHAM

Residence: Hopkinton, MA
(city and either US state, if applicable, or country)

Mailing Address: c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807: UNITED STATES OF AMERICA

Inventor's Signature _____ Date _____
(The signature must be that of the inventor, not that of the agent)

Name:

Residence:
(city and either US state, if applicable, or country)

Mailing Address:

Inventor's Signature _____ Date _____
(The signature must be that of the inventor, not that of the agent)

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

ADVANCE E-MAIL

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92*bis*.1 and Administrative Instructions, Section 422)

To:

DONAHUE, Brian, A.
Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing *(day/month/year)*
20 July 2017 (20.07.2017)

Applicant's or agent's file reference
159792010340

IMPORTANT NOTIFICATION

International application No.
PCT/US2016/013947

International filing date *(day/month/year)*
19 January 2016 (19.01.2016)

1. The following indications appeared on record concerning:

☐ the applicant ☒ the inventor ☐ the agent ☐ the common representative

Name and Address
BURBHAM, Brenda
c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, NJ 08807
United States of America

State of Nationality | State of Residence

Telephone No.

Facsimile No.

E-mail address

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☒ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address
BUNRHAM, Brenda
c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, NJ 08807
United States of America

State of Nationality | State of Residence

Telephone No.

Facsimile No.

E-mail address
☐ Notifications by e-mail authorized

3. Further observations, if necessary:

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

☒ the receiving Office
☒ the International Searching Authority
☐ the Authority(ies) specified for supplementary search
☐ the International Preliminary Examining Authority
☒ the designated Offices concerned
☐ the elected Offices concerned
☐ other:

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

Facsimile No. +41 22 338 89 70

Authorized officer
Mondon Catherine
e-mail pct.team6@wipo.int
Telephone No. +41 22 338 74 06

Form PCT/IB/306 (January 2009) 1/PNG4PLP6H5AX60

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

Application Number
PCT/US2016/013947

TITLE: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

APPLICANT: GENZYME CORPORATION

IPC CLASSIFICATION: C12N7/02, G01N9/30

EXAMINER: Roscoe, Richard

CONSULTED DATABASES: BIOSIS, EMBASE, EPODOC, MEDLINE, NPL, WPI, XPESP

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: C12N7/02, G01N9/30

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
Analytical ultracentrifugation for assessing various aspects of virus preparation purity, in particular in recmobinant adenovirus.

EPO FORM P04A42

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 July 2016 (28.07.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/118520 A1

(51) International Patent Classification:
C12N 7/02 (2006.01) *G01N 9/30* (2006.01)

(21) International Application Number:
PCT/US2016/013947

(22) International Filing Date:
19 January 2016 (19.01.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/105,714 20 January 2015 (20.01.2015) US

(71) Applicant: **GENZYME CORPORATION** [US/US]; 500 Kendall Street, Cambridge, MA 02142 (US).

(72) Inventors: **O'RIORDAN, Catherine, R.**; c/o Sanofi, 55 Corporate Drive, Mail Code: 55A-505A, Bridgewater, NJ 08807 (US). **BURBHAM, Brenda**; c/o Sanofi, 55 Corporate Drive, Mail Code: 55A-505A, Bridgewater, NJ 08807 (US).

(74) Agents: **DONAHUE, Brian, A.** et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

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

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

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

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

(54) Title: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

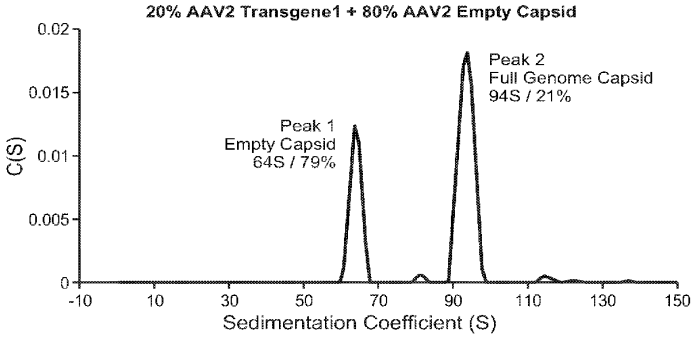


FIG. 1B

(57) Abstract: Provided herein are methods to characterize preparations of recombinant viral particles using analytical ultracentrifugation. Recombinant viral particles include recombinant adeno- associated viral particles, recombinant adenoviral particles, recombinant lentiviral particles and recombinant herpes simplex virus particles. Variant species of recombinant viral particles including empty capsids and recombinant viral particles with variant genomes (e.g., truncated genomes, aggregates, recombinants) can be identified and quantitated. The methods can be used to characterize preparations of recombinant viral particles regardless of the sequence of the recombinant viral genome or the serotype of the recombinant viral capsid.

WO 2016/118520 A1

756755

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

January 30, 2016

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *62/105,714*
FILING DATE: *January 20, 2015*
RELATED PCT APPLICATION NUMBER: *PCT/US16/13947*

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US62/105,714*

Certified by

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 89300

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter to:

[X] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

GENZYME CORPORATION

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	/Andrea Q. Ryan/	Date (Optional)	July 23, 2014
Name	Andrea Q. Ryan		
Title	Attorney-In-Fact		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[X] Total of 1 forms are submitted.

sf-3407816

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159793010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Catherine		O'RIORDAN	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Waban	State/Province	MA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	c/o Sanofi		
Address 2	55 Corporate Drive		
City	Bridgewater	State/Province	NJ
Postal Code	08807	Country i	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Brenda		BURHAM	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Hopkinton	State/Province	MA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	c/o Sanofi		
Address 2	55 Corporate Drive		
City	Bridgewater	State/Province	NJ
Postal Code	08807	Country i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

PTO/AIA/14 (12-13)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159793010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

☐ An Address is being provided for the correspondence Information of this application.			
Customer Number	89300		
Email Address	PatentDocket@mofo.com	Add Email	Remove Email

Application Information:

Title of the Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES		
Attorney Docket Number	159793010300	**Small Entity Status Claimed**	☐
Application Type	Provisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	15	**Suggested Figure for Publication (if any)**	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	89300		

PTO/AIA/14 (12-13)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159793010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the application number blank.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) [i]the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country [i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

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

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (12-13)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159793010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Authorization to Permit Access:

☐ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :	
If the Applicant is an Organization check here. ☒	
Organization Name	Genzyme Corporation

PTO/AIA/14 (12-13)
Approved for use through 01/31/2014. OMB 0651-0032
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 contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159793010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Mailing Address Information:			
Address 1	500 Kendall Street		
Address 2			
City	Cambridge	**State/Province**	MA
Country i	US	Postal Code	02142
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

PTO/AIA/14 (12-13)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	159793010300
	Application Number	
Title of Invention	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	

Signature:

Remove

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications

Signature	/Brian A. Donahue/			Date (YYYY-MM-DD)	2015-01-20
First Name	Brian A.	Last Name	Donahue	Registration Number	58206

Additional Signature may be generated within this form by selecting the Add button. Add

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

~~Express Mail Label No.~~ N/A

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Catherine	O'RIORDAN	Waban, MA
Brenda	BURHAM	Hopkinton, MA

Additional inventors are being named on the _____ separately numbered sheets attached hereto.

TITLE OF THE INVENTION (500 characters max):

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

[x] The address corresponding to Customer Number: 89300

OR

[ ] Firm or Individual Name

Address

City	State	Zip
Country	Telephone	Email

ENCLOSED APPLICATION PARTS (*check all that apply*)

[x] Application Data Sheet. See 37 CFR 1.76. (7 pgs.)
[ ] CD(s), Number of CDs _____
[x] Drawing(s) *Number of Sheets* 15
[x] Other (specify) 1. Power of Attorney (1 pg.)
[x] Specification (e.g. description of the invention) *Number of Pages* 90

Fees Due: Filing Fee of $260 ($130 for small entity) ($65 for micro entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which 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).

METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant asserts small entity status. See 37 CFR 1.27.
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[ ] A check or money order made payable to the *Director of the United States Patent and Trademark Office* is enclosed to cover the filing fee and application size fee (if applicable).
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I hereby certify that this paper is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: January 20, 2015 Signature: /Juan Petris/ (Juan Petris)

sf-3496448

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PROVISIONAL APPLICATION COVER SHEET - Page 2 of 2

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.

[ ] Yes, the invention was made by an agency of the United States Government. The U.S. Government agency name is:

[ ] Yes, the invention was made under a contract with an agency of the U.S. Government. The name of the U.S. Government agency and the Government contract number are:

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SIGNATURE	/Brian A. Donahue/	DATE	January 20, 2015
TYPED or PRINTED NAME	Brian A. Donahue	REGISTRATION NO. *(if appropriate)*	58,206
TELEPHONE	(650) 813-5632	DOCKET NUMBER	159793010300

sf-3496448

Electronic Acknowledgement Receipt	
EFS ID:	21259818
Application Number:	62105714
International Application Number:	
Confirmation Number:	3802
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Juan Petris
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	159793010300
Receipt Date:	20-JAN-2015
Filing Date:	
Time Stamp:	22:24:21
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$260
RAM confirmation Number	8581
Deposit Account	031952
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Provisional Cover Sheet (SB16)	15979-3010300_Transmittal.pdf	39568 33364f8a550e5dae15629b2272d64a1826695f1d	no	2
Warnings:					
This is not a USPTO supplied Provisional Cover Sheet SB16 form.					
Information:					
2	Power of Attorney	15979-3010300_POA.pdf	114513 17d905aa42c1c4609b05e23c9e6ccf04841710b8	no	1
Warnings:					
Information:					
3	Application Data Sheet	15979-3010300_ADS.PDF	1560753 0dd81715abc265b550b9abfe0eb20390a51aefce	no	7
Warnings:					
Information:					
4		15979-3010300_Specification_Claims_Abstract.pdf	317955 36003fa6b51e99b2bee1a00be00a8001e8306633	yes	90

Multipart Description/PDF files in .zip description

Document Description	Start	End
Specification	1	75
Claims	76	89
Abstract	90	90

Warnings:					
Information:					
5	Drawings-only black and white line drawings	15979-3010300_Figures.pdf	2681602 613f3a30315d38ffb1e91c6c30c8f87230774 5d59	no	15
Warnings:					
Information:					

6	Fee Worksheet (SB06)	fee-info.pdf	30149 feafa234a9ef3547cb7f75982def0c8ac086a682	no	2
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			Total Files Size (in bytes):	4744540	

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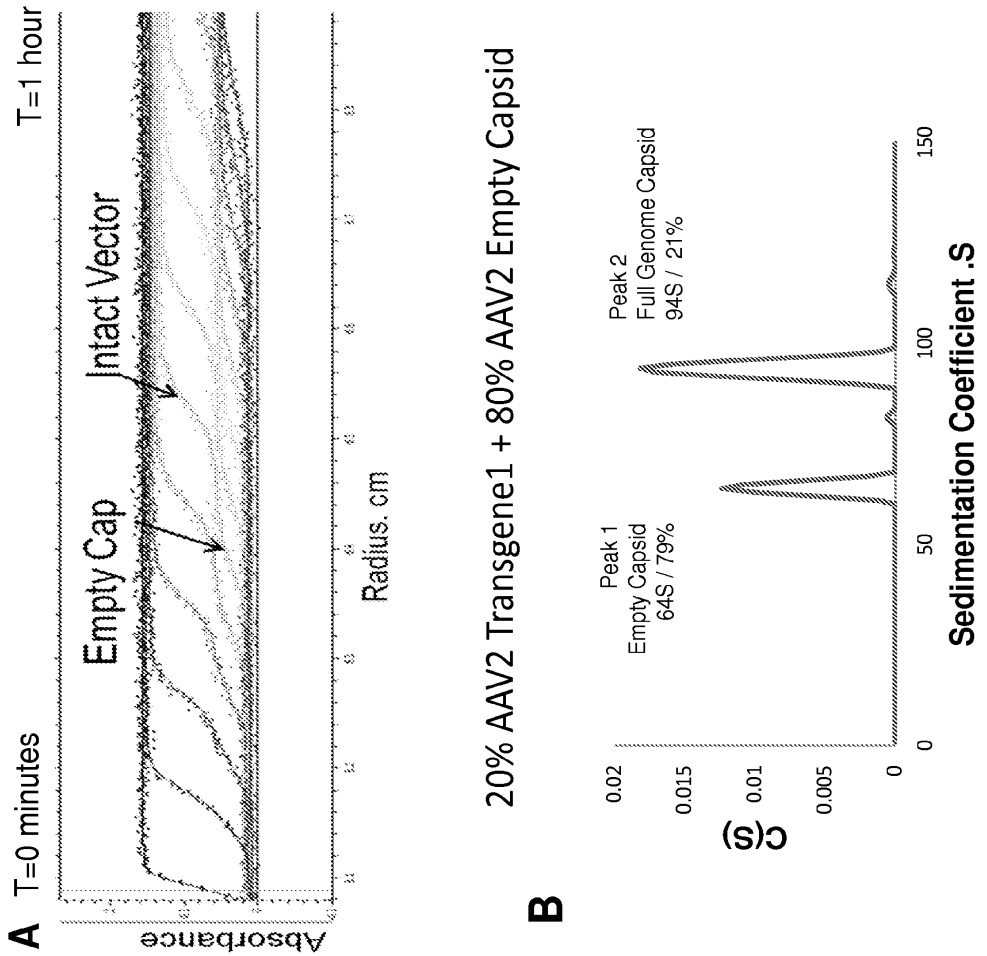

A
T=0 minutes
T=1 hour
Empty Cap
Intact Vector
Absorbance
Radius. cm
B
20% AAV2 Transgene1 + 80% AAV2 Empty Capsid
Peak 1
Empty Capsid
64S / 79%
Peak 2
Full Genome Capsid
94S / 21%
c(s)
0.02
0.015
0.01
0.005
0
0
50
100
150
Sedimentation Coefficient .S

FIG. 1

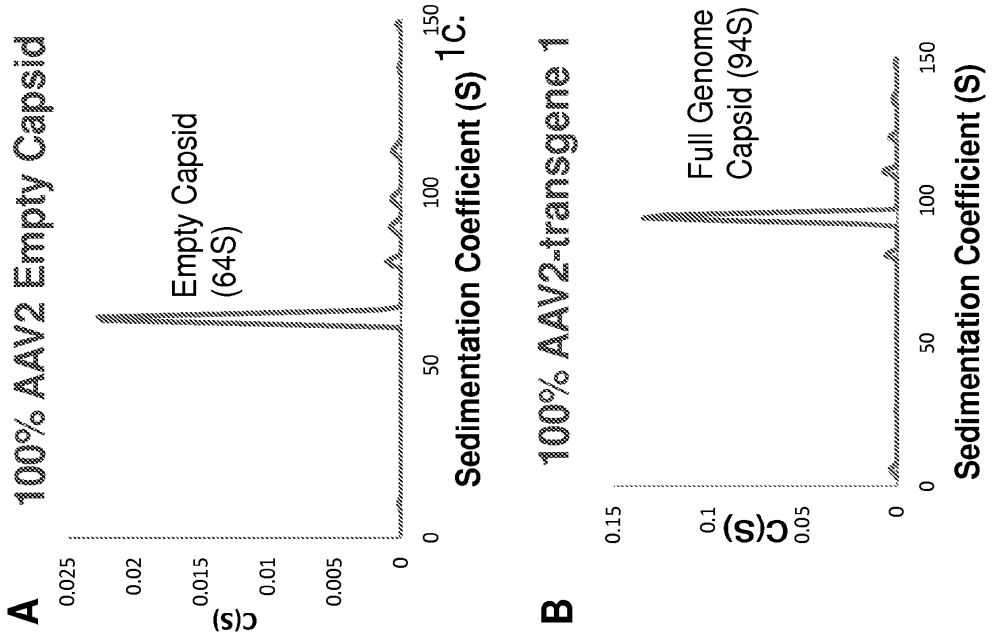

A
100% AAV2 Empty Capsid
Empty Capsid
(64S)
c(s)
0.025
0.02
0.015
0.01
0.005
0
0
50
100
150
Sedimentation Coefficient (S)
B
100% AAV2-transgene 1
Full Genome
Capsid (94S)
c(s)
0.15
0.1
0.05
0
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2

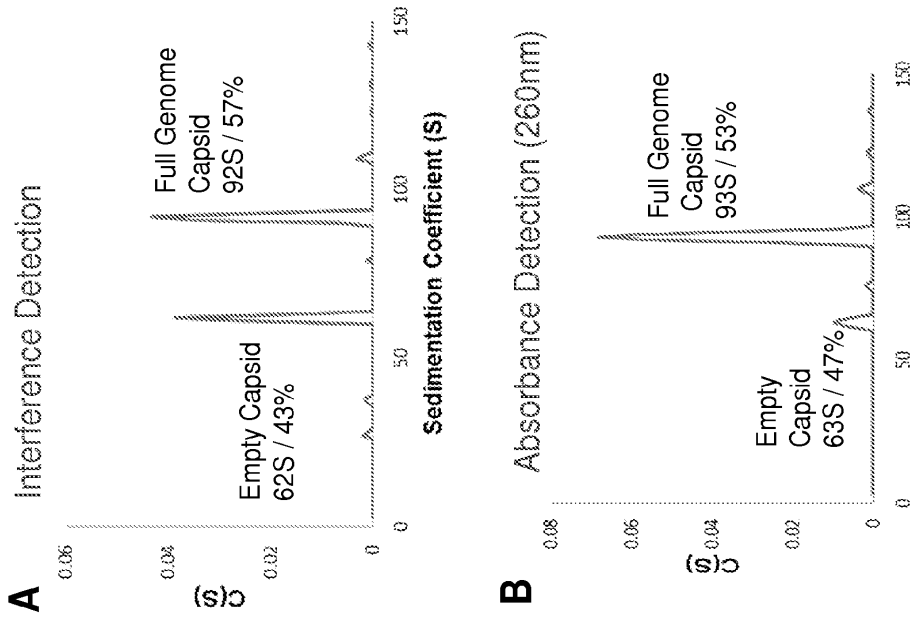

A
Interference Detection
c(s)
0.06
0.04
0.02
0
Empty Capsid
62S / 43%
Full Genome
Capsid
92S / 57%
0
50
100
150
Sedimentation Coefficient (S)
B
Absorbance Detection (260nm)
c(s)
0.08
0.06
0.04
0.02
0
Empty
Capsid
63S / 47%
Full Genome
Capsid
93S / 53%
0
50
100
150
Sedimentation Coefficient. S

FIG. 3

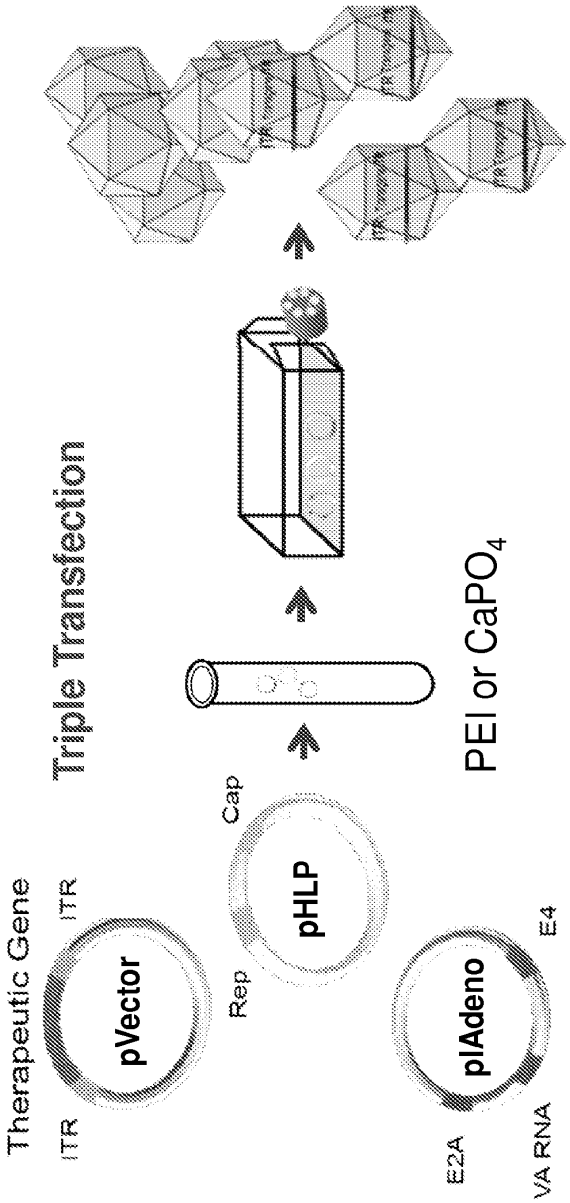

Therapeutic Gene
ITR
ITR
pVector
Cap
Rep
pHLP
pIAdeno
E4
E2A
VA RNA
PEI or $CaPO_4$
Triple Transfection

FIG. 4

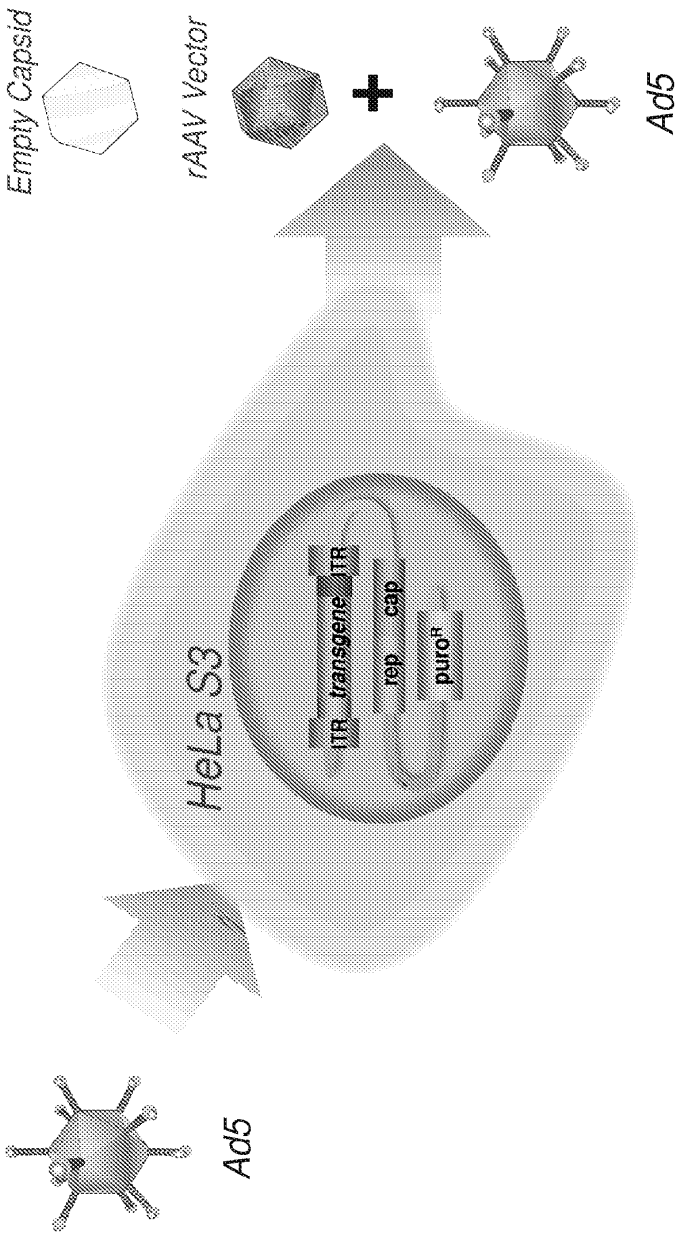

Empty Capsid
rAAV Vector
Ad5
HeLa S3
ITR
transgene
ITR
rep
cap
puroR
Ad5

FIG. 5

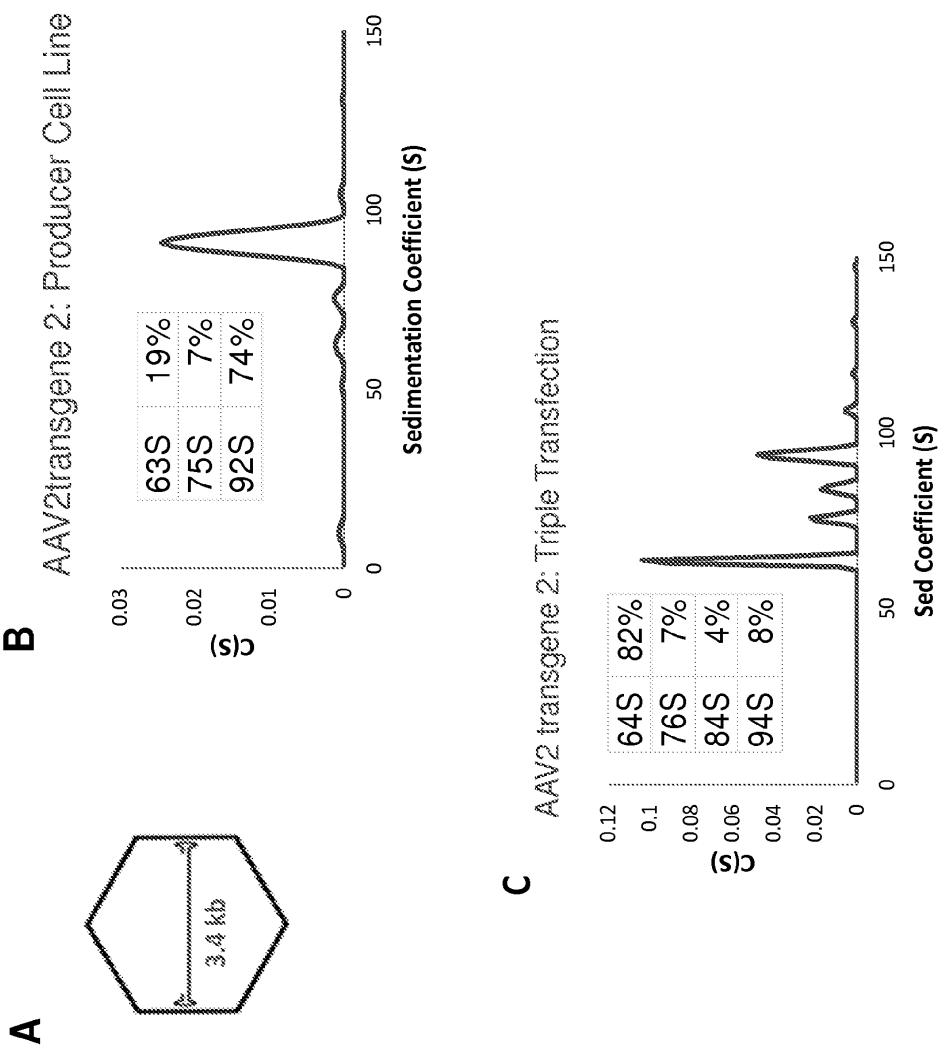

A
3.4 kb
B
AAV2transgene 2: Producer Cell Line
63S 19%
75S 7%
92S 74%
c(s)
0.03
0.02
0.01
0
0
50
100
150
Sedimentation Coefficient (S)
C
AAV2 transgene 2: Triple Transfection
64S 82%
76S 7%
84S 4%
94S 8%
c(s)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
50
100
150
Sed Coefficient (S)

FIG. 6

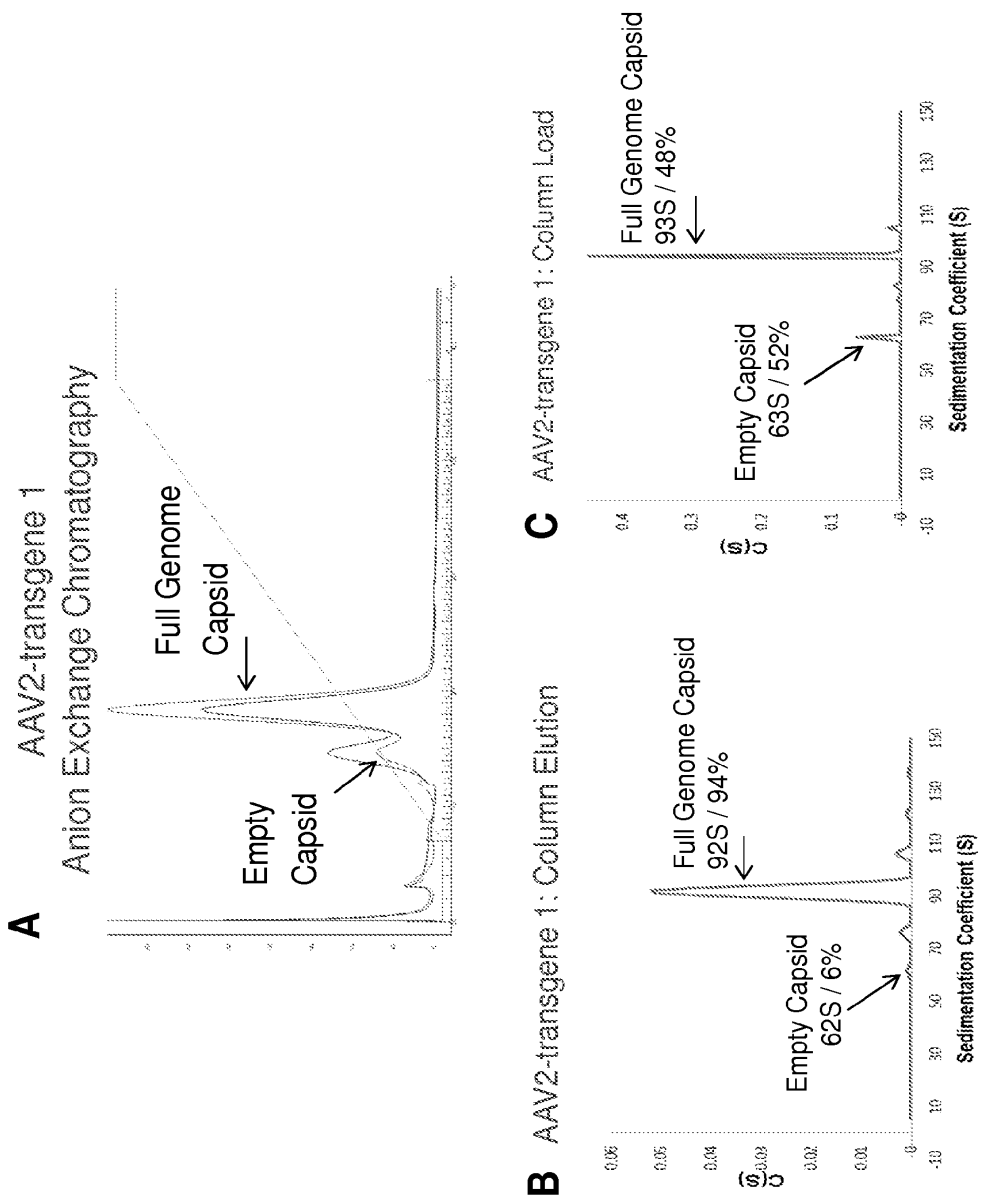

A
AAV2-transgene 1
Anion Exchange Chromatography
Full Genome
Capsid
Empty
Capsid
B
AAV2-transgene 1: Column Elution
Full Genome Capsid
92S / 94%
Empty Capsid
62S / 6%
c(s)
Sedimentation Coefficient (S)
C
AAV2-transgene 1: Column Load
Full Genome Capsid
93S / 48%
Empty Capsid
63S / 52%
c(s)
Sedimentation Coefficient (S)

FIG. 7

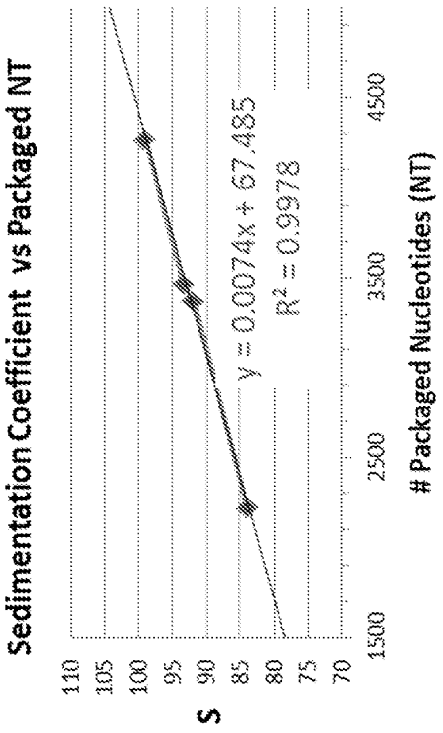

Sedimentation Coefficient vs Packaged NT
S
110
105
100
95
90
85
80
75
70
1500
2500
3500
4500
Packaged Nucleotides (NT)
y = 0.0074x + 67.485
R² = 0.9978

FIG. 8

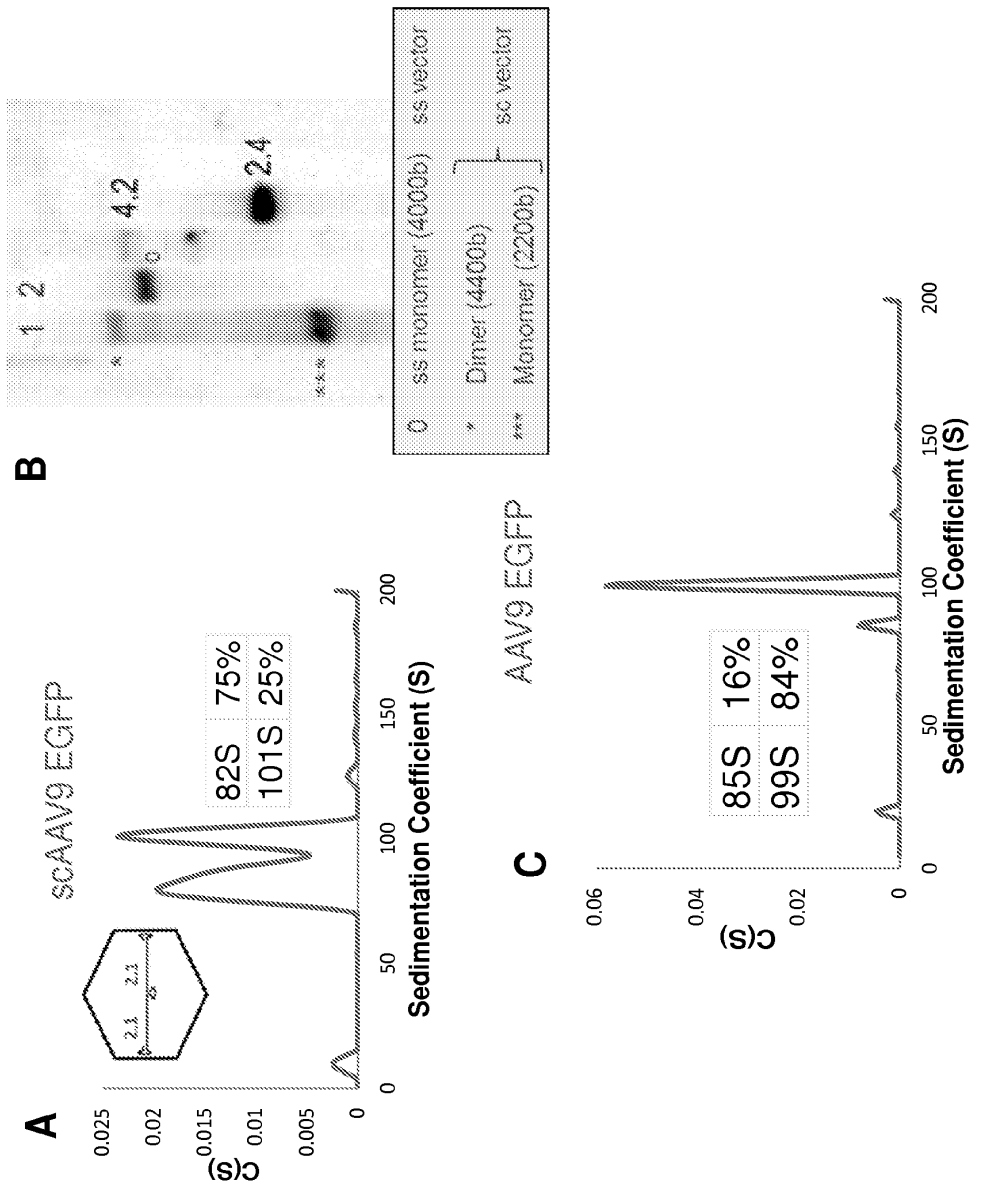

A
scAAV9 EGFP
82S 75%
101S 25%
c(s)
Sedimentation Coefficient (S)
B
1 2
4.2
2.4
0 ss monomer (4000b) ss vector
* Dimer (4400b)
*** Monomer (2200b) sc vector
C
AAV9 EGFP
85S 16%
99S 84%
c(s)
Sedimentation Coefficient (S)

FIG. 9

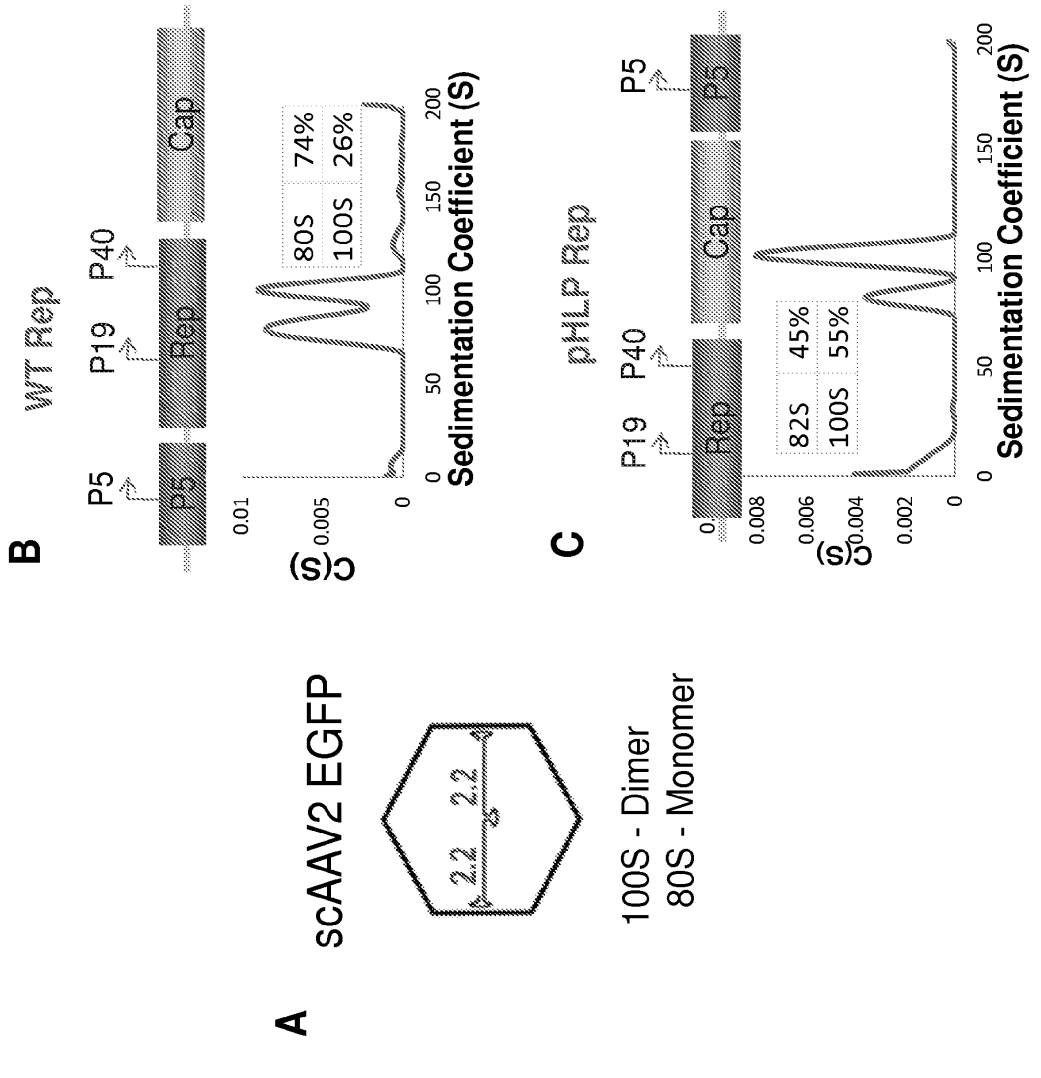

A
scAAV2 EGFP
2.2
2.2
100S - Dimer
80S - Monomer
B
WT Rep
P5
P19
P40
P5
Rep
Cap
80S 74%
100S 26%
c(s)
Sedimentation Coefficient (S)
C
pHLP Rep
P5
P19
P40
P5
Rep
Cap
P5
82S 45%
100S 55%
c(s)
Sedimentation Coefficient (S)

FIG. 10

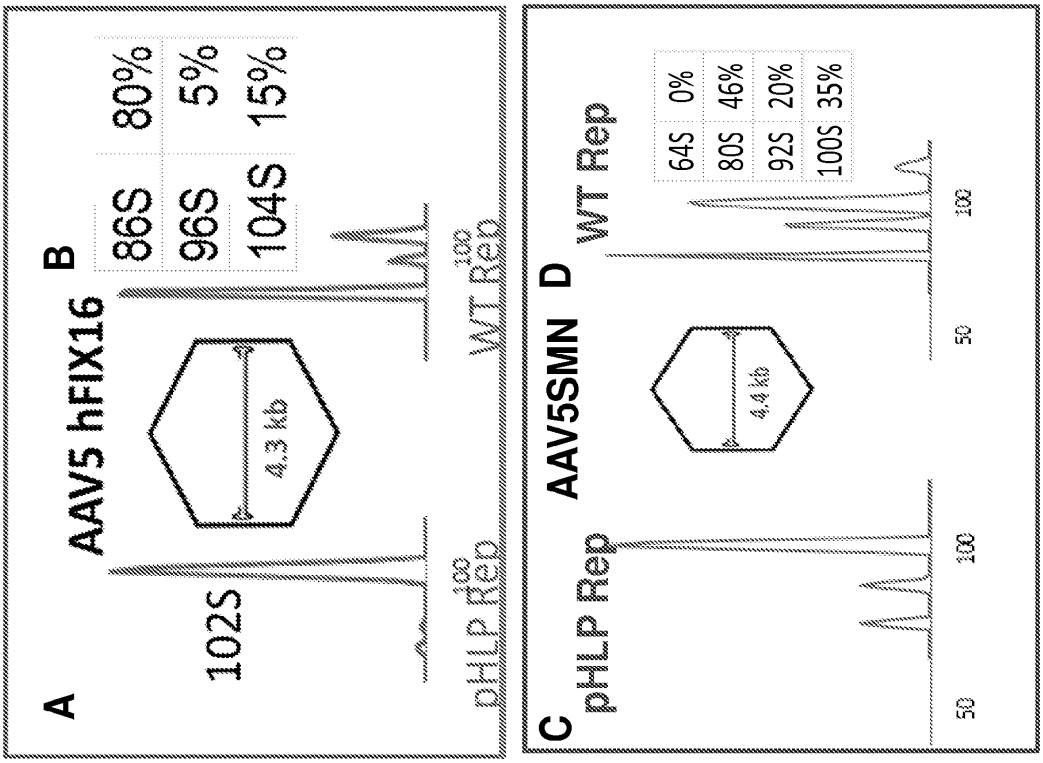

A
AAV5 hFIX16
B
86S 80%
96S 5%
104S 15%
102S
4.3 kb
pHLP Rep 100
WT Rep 100
C
pHLP Rep
AAV5SMN
D
WT Rep
64S 0%
80S 46%
92S 20%
100S 35%
4.4 kb
50
100
50
100

FIG. 11

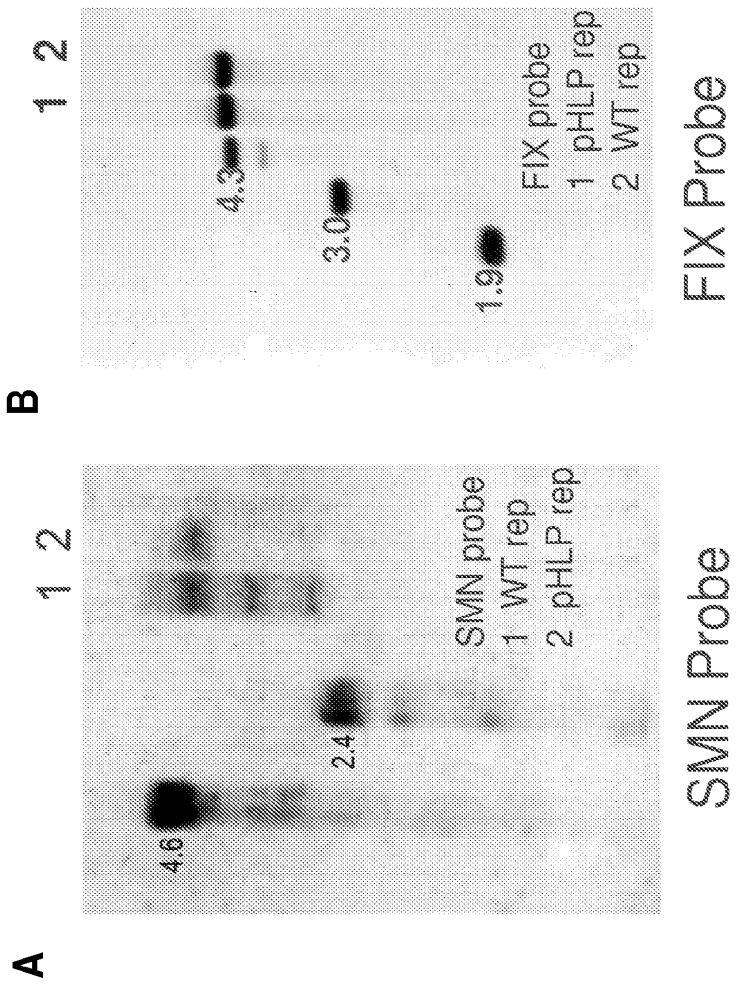

A
1 2
4.6
2.4
SMN probe
1 WT rep
2 pHLP rep
SMN Probe
B
1 2
4.3
3.0
1.9
FIX probe
1 pHLP rep
2 WT rep
FIX Probe

FIG. 12

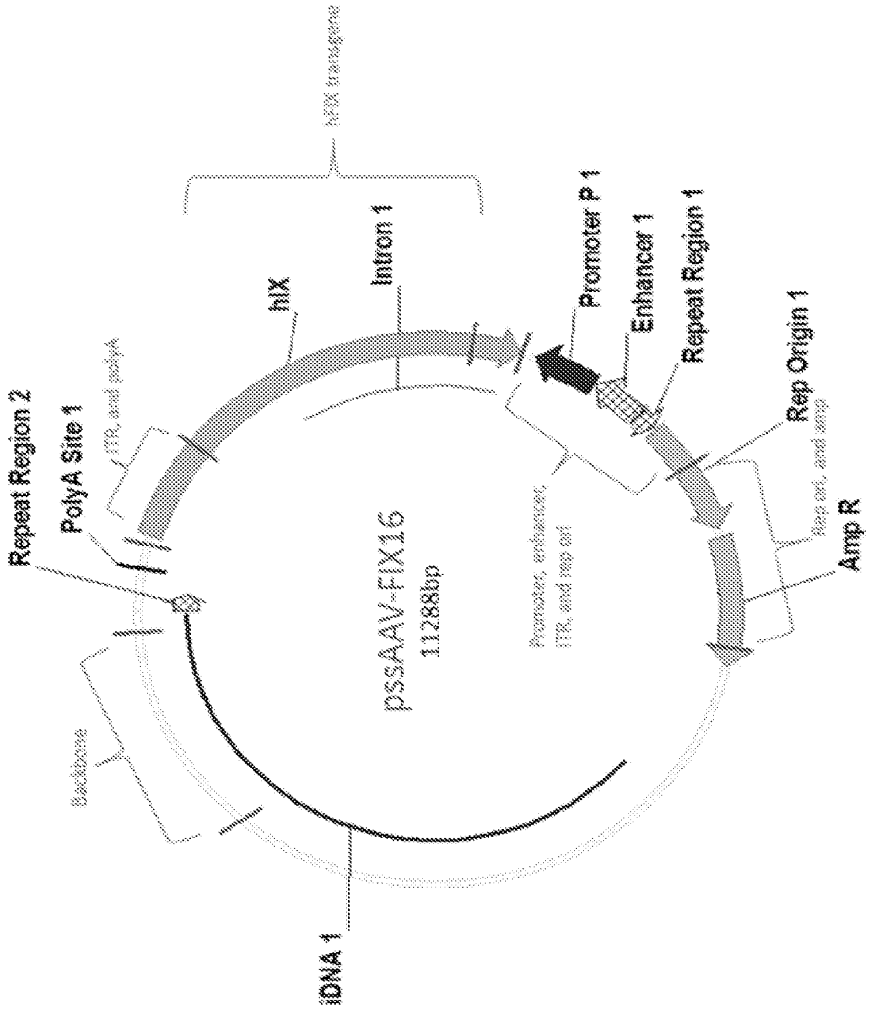

hFIX transgene
Repeat Region 2
PolyA Site 1
ITR and polyA
hIX
Intron 1
Promoter P 1
Enhancer 1
Repeat Region 1
Rep Origin 1
Amp R
pssAAV-FIX16
11288bp
Promoter, enhancer, ITR, and rep ori
Rep ori, and amp
Backbone
iDNA 1

FIG. 13

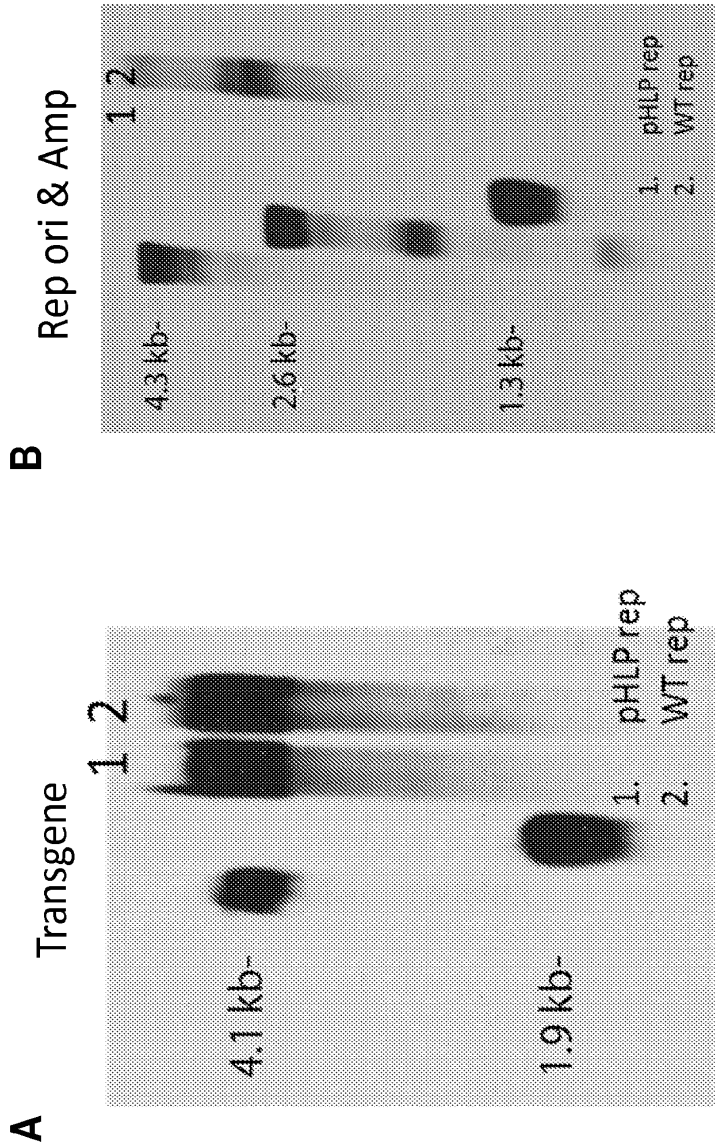

A
Transgene
1 2
4.1 kb-
1.9 kb-
1. pHLP rep
2. WT rep
B
Rep ori & Amp
1 2
4.3 kb-
2.6 kb-
1.3 kb-
1. pHLP rep
2. WT rep

FIG. 14

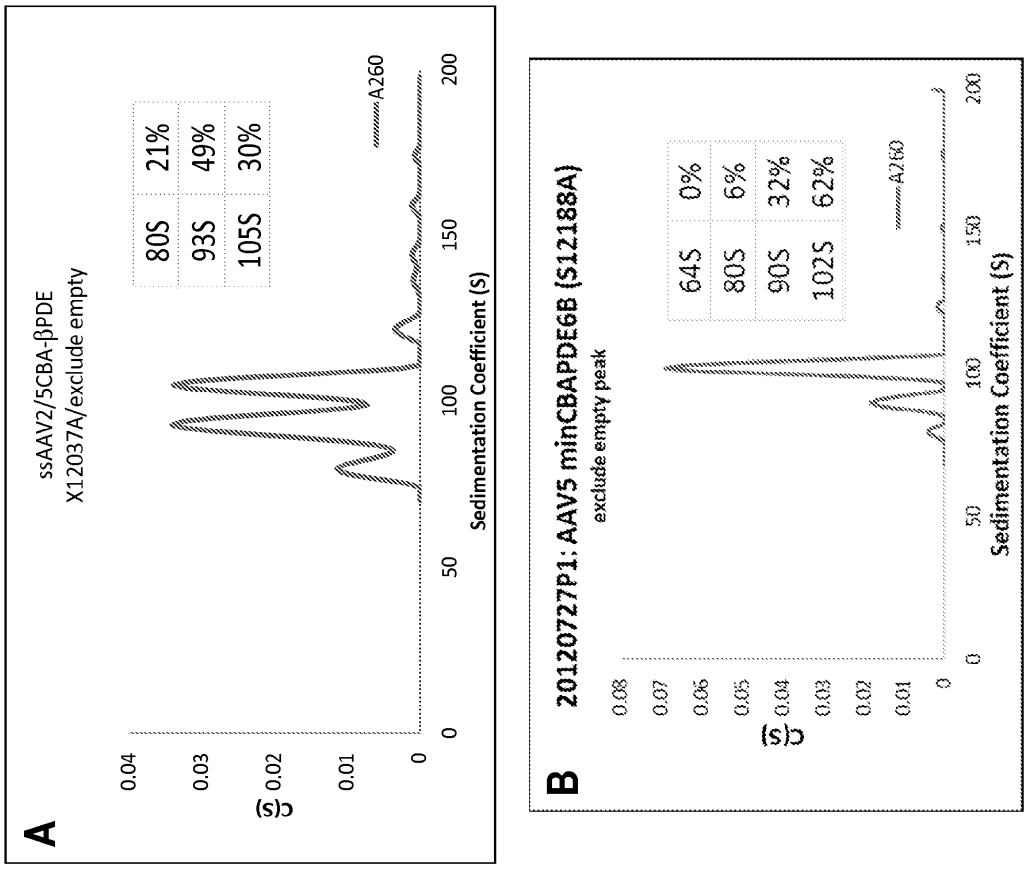

A
ssAAV2/5CBA-βPDE
X12037A/exclude empty
80S 21%
93S 49%
105S 30%
A260
c(s)
Sedimentation Coefficient (S)
B
20120727P1: AAV5 minCBAPDE6B (S12188A)
exclude empty peak
64S 0%
80S 6%
90S 32%
102S 62%
A260
c(s)
Sedimentation Coefficient (S)

FIG. 15

ABSTRACT

Provided herein are methods to characterize preparations of recombinant viral particles using analytical ultracentrifugation. Recombinant viral particles include recombinant adeno-associated viral particles, recombinant adenoviral particles, recombinant lentiviral particles and recombinant herpes simplex virus particles. Variant species of recombinant viral particles including empty capsids and recombinant viral particles with variant genomes (*e.g.*, truncated genomes, aggregates, recombinants) can be identified and quantitated. The methods can be used to characterize preparations of recombinant viral particles regardless of the sequence of the recombinant viral genome or the serotype of the recombinant viral capsid.

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

FIELD OF THE INVENTION

[0001] The present invention relates to methods to characterize recombinant viral vectors; *e.g.*, recombinant adeno-associated viral (AAV) particles, recombinant adenoviral (rAd) particles, recombinant lentiviral particles and recombinant Herpes simplex viral (rHSV) particles using analytical ultracentrifugation.

BACKGROUND OF THE INVENTION

[0002] Recombinant viruses show great promise and utility as a vehicle to deliver therapeutic nucleic acids for gene therapy applications. A number of different recombinant viruses are used in these gene therapy applications based on a number of factors including the size of the nucleic acid to be delivered, the target cell or tissue to deliver the nucleic acid, the need for short or long term expression of the therapeutic nucleic acid, and integration of the therapeutic nucleic acid into the recipient's genome. Examples of viruses used in gene therapy applications include adeno-associated virus (AAV), adenovirus, lentivirus and herpes simplex virus (HSV).

[0003] The generation of recombinant viral vectors for the clinic requires an analytical method that monitors drug product quality with regard to homogeneity, purity and consistency of manufacturing, yet to date no method to support such a characterization has been established. Typically, the DNA content of recombinant viral DNA viral vectors is measured by Southern blot analysis using a sequence specific probe. Viral capsids or envelopes may be characterized by immunoassay using an antibody that binds specifically to a capsid or envelope protein of a particular recombinant virus. For example, Steinbach, S *et al.*, (1997) *J. Gen. Virol.*, 78:1453-1462 provides an immunoassay for rAAV serotypes. What is needed is a generic assay to characterize recombinant viral preparations regardless of the nucleic acid sequence of the recombinant viral genome or the serotype of the capsid.

[0004] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0005] In some aspects, the invention provides methods of characterizing a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

[0006] In some aspects, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

[0007] In some aspects, the invention provides methods to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and b) plotting the

differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

[0008] In some aspects, the invention provides, methods of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, and d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

[0009] In some aspects, the invention provides methods of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

[0010] In some aspects, the invention provides methods of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

[0011] In some aspects, the invention provides methods of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

[0012] In some aspects, the invention provides, methods of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method

of any one of claims 5-8, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles. In some embodiments, the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles. In some embodiments, the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes. In some embodiments, the capsid particles comprising variant sized genomes comprise truncated genomes, aggregates, recombinants and/or DNA impurities.

[0013] In some aspects, the invention provides methods of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation. In some embodiments, the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes. In some embodiments, the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles.

[0014] In some aspects, the invention provides methods of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the above method, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes

indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

[0015] In some embodiments of the above aspects, sedimentation of recombinant viral particles is monitored by absorbance. In some embodiments, the absorbance is at about 230 nm, 260 nm or 280 nm. In some embodiments, the absorbance is at about 260 nm. In some embodiments, sedimentation of recombinant viral particles is monitored by interference. In some embodiments, the interference is Rayleigh interference.

[0016] In some embodiments of the above aspects, the preparation is an aqueous solution. In further embodiments, the aqueous solution comprises a pharmaceutical formulation. In some embodiments, the aqueous solution comprises a buffer. In some embodiments, the buffer is at physiological pH. In some embodiments, the buffer is at physiological osmolality. In some embodiments, the pharmaceutical formulation comprises phosphate buffered saline (PBS). In some embodiments, the PBS has pH of about 7.2 and an osmolality of about 300 mOsm/L. In some embodiments, the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

[0017] In some embodiments of the above aspects, the C(S) values are determined by an algorithm that comprises Lamm equation solutions. In some embodiments, the algorithm is the SEDFIT algorithm. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge; for example, the sector may be a portion of the ultracentrifuge comprising a detection system. In some embodiments, the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until recombinant viral particles sediment to the bottom of ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window.

[0018] In some embodiments, the radial concentration is recorded for at least about any of 0.5 hours, 0.75 hours, 1.0 hours, 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, or 5.0 hours. In some embodiments, the radial concentration is recorded for about 1.0 hour. In some

embodiments, the radial concentration is recorded for about 1.2 hours. In some embodiments, the radial concentration is recorded from about 0.5 hours to about 2.0 hours. In some embodiments, the radial concentration is recorded from about 1.0 hours to about 2.0 hours.

[0019] In some embodiments of the above aspects, at least 30 scans are used to monitor sedimentation of recombinant viral particles. In some aspects, about 30 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 30 to about 75 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 30 to about 50 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 50 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

[0020] In some embodiments of the above aspects, a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68. In some embodiments, the regularization is a second derivative regularization. In some embodiments, the regularization is Max entropy regularization. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.90. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.99. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68.

[0021] In some embodiments of the above aspects, the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software. In some embodiments, resolution is about 200S to about 1000S. In some embodiments, resolution is about 200S. In some embodiments, S min is about 1. In some embodiments, Smax is about 100S to about 1000S. In other embodiments, Smax is about 200S to about 5000S. In other embodiments, Smax is about 200S. In some embodiments, the frictional ratio is left to float to a value determined by centrifugation software. In some embodiments, the frictional ratio is about 1.0. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

[0022] In some embodiments of the above aspects, the sedimentation of recombinant viral particles is monitored about every 10-60 seconds. In some embodiments, sedimentation of recombinant viral particles is monitored (*e.g.*, scanned) about every 10 seconds. In other embodiments, the sedimentation of recombinant viral particles is monitored about every 60 seconds. In some embodiments, the sedimentation velocity of recombinant viral during ultracentrifugation is determined by monitoring the sedimentation of recombinant viral particles once in more than about every 15 seconds, 30 seconds, 45 seconds, 1 minute (60 seconds), 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes.

[0023] In some embodiments of the above aspects, the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm. In some embodiments, the boundary sedimentation velocity is performed at about 3,000 rpm to about 10,000 rpm. In other embodiments, the boundary sedimentation velocity is performed at about 10,000 rpm to about 20,000 rpm. In other embodiments, the boundary sedimentation velocity is performed at about 15,000 rpm to about 20,000 rpm.

[0024] In some embodiments of the above aspects, the boundary sedimentation velocity is performed at about 4°C to about 20 °C. In some embodiments, the boundary sedimentation velocity is performed at about 4°C.

[0025] In some embodiments of the above aspects, the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle. In some embodiments, the recombinant viral particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the recombinant viral particle comprises an AAV1 ITR, an AAV2 ITR,

an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In other embodiments, the recombinant viral particle is a recombinant adenoviral particle. In some embodiments, the recombinant adenoviral particle comprises an capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, or porcine Ad type 3. In some embodiments, the recombinant adenoviral particle comprises a variant of an adenovirus serotype 2 capsid or a variant of an adenoviral serotype 5 capsid. In other embodiments, the recombinant viral particle is a recombinant lentiviral particle. In some embodiments, the recombinant lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114 or variants therein. In other embodiments, the recombinant viral particle is a rHSV particle. In some embodiments, the HSV particle is an HSV-1 particle or an HSV-2 particle.

[0026] In some aspects, the invention provides methods for evaluating a process for the production of recombinant viral particles comprising the method of any one of claims 1 to 67, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles. In some embodiments, the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle. In some embodiments, the rAAV particles are produced from a producer cell line. In other embodiments, the rAAV particles are produced by triple transfection of i) nucleic acid encoding AAV rep and cap, ii) rAAV vector sequences, and iii) nucleic acid encoding adenovirus helper functions. In other embodiments, the recombinant viral particles are produced by an AAV/HSV hybrid. In other embodiments, the recombinant viral particles are

produced from a baculovirus cell. In some embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell. In some embodiments, the recombinant viral particles are produced by introduction of one or more nucleic acids encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell, wherein the one or more nucleic acids are introduced to the cell using a recombinant helper virus. In some embodiments, the recombinant helper virus is an adenovirus or a herpes simplex virus. In some embodiments, the recombinant viral particles comprise a self-complementary AAV (scAAV) genome. In some embodiments, the method is used to detect the presence of recombinant viral particles comprising the monomeric form of a scAAV genome or the dimeric form of a scAAV genome.

[0027] In some embodiments of the above aspect, the recombinant viral particles are produced by transient transfection of nucleic acid encoding adenovirus vector sequences and adenovirus replication and packaging sequences to a suitable host cell. In other embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding lentivirus vector sequences and/or lentivirus replication and packaging sequences to a suitable host cell. In other embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding HSV vector sequences and/or HSV replication and packaging sequences to a suitable host cell.

[0028] In some aspects the invention provides methods for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release recombinant viral particles; c) isolating the recombinant viral particles produced by the host cell; and d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the above methods. In some aspects the invention provides methods for preparing recombinant viral particles with

reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release recombinant viral particles; c) isolating the recombinant viral particles produced by the host cell; and d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the above methods. In some embodiments, the p5 promoter is located 3' to the rep and/or cap coding region. In some embodiments, the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

[0029] In some embodiments, of any of the preceding embodiments, the recombinant viral particles have been purified using one or more purification steps.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] **FIG. 1** shows that analytical ultracentrifugation (AUC) can be used to characterize recombinant viral vector particles. (**A**) A representative scanning profile of boundary sedimentation velocity depicting the absorbance (260 nm) versus the radius (cm) of an AAV2 mixture over a time interval (T) of 1.2 hours. The AAV2 mixture contained empty capsids ("Empty Cap") and full genome capsids ("Intact Vector"). (**B**) A plot of concentration in units of detection, C(S), versus sedimentation coefficient (Svedberg units, S) showing that AUC can be used to measure the concentration of empty capsids and full genome capsids from an 80%/20% mixture. Each peak is labeled with the particle species and its corresponding sedimentation coefficient (S) and relative abundance (%).

[0031] **FIG. 2** shows the AUC profiles of pure populations of empty AAV2 capsids (A) and genome-containing AAV2-transgene 1 capsids (B). Each peak is labeled with the capsid species and its sedimentation coefficient (S).

[0032] **FIG. 3** shows a comparison between interference and absorbance detection methods by AUC. (**A**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a 1:1 mixture of empty and genome-containing capsids generated using interference optical detection. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a 1:1 mixture of empty and genome-containing capsids generated using absorbance optical detection (260 nm). The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0033] **FIG. 4** illustrates the triple transfection method for AAV vector production. The three vectors, containing the gene of interest ("pVector"), AAV Rep and Cap genes ("pHLP"), and adenoviral components ("pIAdeno") are labeled. Note that both genome-containing (labeled with the "ITR-Transgene-ITR" graphic) and empty capsids (blank) are produced.

[0034] **FIG. 5** illustrates the producer cell line method for AAV vector production. As labeled, the HeLa S3 cell line contains integrated Rep, Cap, and Puromycin resistance genes, along with an ITR-flanked transgene of interest. This cell line is infected with adenovirus ("*Ad5*") to stimulate recombinant viral production. Note that both genome-containing (labeled "recombinant viral Vector") and empty capsids are produced, in addition to adenovirus particles.

[0035] **FIG. 6** shows that vector production by the producer cell line and triple transfection methods yields different vector preparations, as revealed by AUC analysis. (**A**) A schematic of the AAV2-transgene 2 vector and its 3.4 kb genome. (**B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation produced by the producer cell line method. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of

sedimentation coefficients for a vector preparation produced by the triple transfection method. The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0036] **FIG. 7** shows that the AUC method may be used to monitor the quality and efficacy of vector purification. (**A**) A plot showing the purification of full-genome AAV2-transgene 1 capsids from empty capsids using anion exchange chromatography. Peak fractions corresponding to each species are labeled. (**B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation after elution from the anion exchange column. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation before chromatography. The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0037] **FIG. 8** shows the linear relationship between sedimentation coefficient and vector genome size. A standard curve plotting sedimentation coefficient (S) versus genome size is depicted, along with a line of best fit, its formula, and its associated R^2 value.

[0038] **FIG. 9** shows that assessment of capsid genome size using AUC data correlates with assessment of genome size by Southern blot. (**A**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV9 EGFP vector preparation. Single stranded monomeric (82S) and double stranded dimeric (101S) species are labeled with the corresponding sedimentation coefficients and relative abundance values (%). A schematic of the vector is also provided. (**B**) Alkaline Southern blot analysis of the DNA from scAAV9 EGFP (lane 1) and single stranded AAV9 EGFP (lane 2) vector capsids. Corresponding bands are labeled as described in the blot legend. 4.2 and 2.4 kb size standards are provided as labeled. (**C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a single stranded AAV9 EGFP vector preparation. 82S and 99S (full genome)

peaks are labeled with the corresponding sedimentation coefficient and relative abundance values (%).

[0039] **FIG. 10** shows that the Rep/Cap promoter position affects genome packaging in recombinant viral vectors produced by the triple transfection method. (**A**) A schematic of the self-complementary scAAV2 EGFP vector, with estimated sedimentation coefficients for the dimeric and monomeric genome species. (**B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV2 EGFP vector preparation produced using a "wild-type" helper plasmid with the endogenous p5 promoter driving Rep 78/68 expression ("WT Rep"). Peaks for single stranded monomeric (80S) and double stranded dimeric (100S) species are labeled with the corresponding relative abundance values (%). (**C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV2 EGFP vector preparation produced using a "wild-type" helper plasmid with the p5 promoter driving Rep 78/68 expression moved downstream of the cap2 sequence ("pHLP Rep"). Peaks for single stranded monomeric (82S) and double stranded dimeric (100S) species are labeled with the corresponding relative abundance values (%).

[0040] **FIG. 11** shows that the Rep/Cap promoter position affects genome packaging in two additional AAV vectors. (**A-B**) Plots of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yield the distribution of sedimentation coefficients for the single stranded AAV5 Factor IX vector (AAV5 hFIX16) containing a cap5 sequence produced with a helper plasmid having an endogenous p5 promoter ("WT Rep," **B**) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," **A**). (**C-D**) Plots of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yield the distribution of sedimentation coefficients for the single stranded AAV5hSMN vector (AAV5SMN) containing a cap5 sequence produced with a helper plasmid having an endogenous p5 promoter ("WT Rep," **D**) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," **C**).

[0041] **FIG. 12** reveals that Southern blot analysis correlates with AUC analysis but misses some fragmented genomes detectable by AUC. (**A**) Southern blot analysis of vector DNA from AAV5SMN preparations made with the pHLP helper plasmid (lane 2) or the WT Rep plasmid (lane 1). 4.6 and 2.4 kb size standards are provided as labeled. (**B**) Southern blot analysis of vector DNA from AAV5FIX preparations made with the pHLP helper plasmid (lane 1) or the WT Rep plasmid (lane 2). 4.3, 3.0, and 1.9 kb size standards are provided as labeled.

[0042] **FIG. 13** provides a map of the AAV5 Factor IX vector indicating the positions of the hFIX transgene, ITR, Rep origin, and *AmpR* marker gene, among other features. Note that the *AmpR* marker is upstream of the ITR, enhancer, and promoter region.

[0043] **FIG. 14** shows that WT Rep vector genomes, unlike pHLP Rep vector genomes, package sequences upstream of the 5' ITR in the AAV5 Factor IX vector. (**A**) Southern blot analysis using an hFIX transgene-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes. (**B**) Southern blot analysis using a Rep ori/*AmpR*-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes.

[0044] **FIG. 15** shows the fragmentation of oversized AAV vector genomes, as demonstrated by AUC analysis. (**A**) Plot of concentration, C(S), versus sedimentation coefficient (S) generated by AUC for an AAV vector with an oversized genome. This genome contains a full-length chicken β-actin (CBA) promoter driving expression of β-phosphodiesterase (ssAAV2/5CBA-βPDE). Peaks for detected species are labeled by observed sedimentation coefficient (S) and relative abundance values (%). (**B**) Plot of concentration, C(S), versus sedimentation coefficient (S) generated by AUC for an AAV vector with a truncated genome. This genome contains a CBA promoter with a reduced-size intron driving expression of β-phosphodiesterase (AAV5 minCBAPDE6B). Peaks for detected species are labeled by observed sedimentation coefficient (S) and relative abundance values (%).

DETAILED DESCRIPTION

[0045] The present invention provides methods of characterizing preparations of viral particles using analytical ultracentrifugation. By subjecting preparations to analytical

ultracentrifugation (AUC) under boundary sedimentation velocity conditions, the sedimentation of viral particles can be monitored at time intervals (*e.g.*, one or more times). The differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S) is then plotted and the area under each peak in the C(s) distribution is integrated to determine the relative concentration of each peak. Each peak represents a species of viral particle reflective of its molecular weight. The species that can be detected by these methods include, but are not limited to, recombinant adeno-associated viral (rAAV) particles, recombinant adenoviral (rAd) particles, recombinant lentiviral particles, and recombinant herpes simplex viral (rHSV) particles. To use rAAV particles as an illustrative example, these methods allow the detection of rAAV species including rAAV capsid particles comprising intact rAAV genomes (*e.g.*, full capsids), empty viral capsids wherein no rAAV genomes have been encapsidated into viral capsids, and rAAV particle variants in which variant rAAV genomes are encapsidated in viral capsids (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates, and the like). These methods can be applied to preparations of viral particles regardless of nucleotide sequence of the viral genome or, in the case of recombinant viral particles, the serotype of the recombinant viral capsid. These methods can be applied to rAAV, rAd, recombinant lentivirus and rHSV viral particles.

[0046] In some aspects, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles by subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times). By plotting the differential sedimentation coefficient distribution value C(S) versus the sedimentation coefficient in Svedberg units (S), species of recombinant viral particles in the preparation can be identified by presence of peaks on the plot corresponding to an S value. The genome size of a particular species of recombinant viral particles can be calculated, for example, by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of different known size. The vector genomes that can be assessed by these methods include, but are not limited to, recombinant viral capsid particles comprising intact recombinant viral genomes (*e.g.*, full capsids), empty viral capsids wherein no recombinant viral genomes have been

encapsidated into viral capsids, and recombinant viral particle variants in which variant recombinant viral genomes (*e.g.,* particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates and the like) are encapsidated in viral capsids. In some embodiments, the viral particles are rAAV, rAd, recombinant lentivirus or rHSV viral particles.

[0047] In some embodiments the invention provides methods of determining the heterogeneity of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) in a preparation of recombinant viral particles by AUC under boundary sedimentation velocity conditions wherein the presence of peaks in a plot of C(S) v. S, in addition to the peak representing capsids comprising an intact viral genome, indicates heterogeneity of recombinant viral particles in the preparation. In some embodiments, the relative amounts of each recombinant viral species in the preparation are calculated by integrating the area for each peak in the plot.

[0048] In some embodiments of the invention, AUC is used to determine the presence of empty capsids and/or recombinant viral particle variants in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles), wherein the presence of peak that corresponds to the S value of empty capsid particles and/or recombinant viral particle variants in a plot of C(S) vs. S indicates the presence of empty capsid particles and/or recombinant viral particle variants. In some embodiments, the relative amount of empty capsids and/or recombinant viral particle variants in a preparation of recombinant viral particles is determined by integrating the area under each peak in a plot of C(S) versus S and comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles and/or recombinant viral particle variants to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value corresponding to empty capsid particles and/or recombinant viral particle variants is compared to the total amount of all recombinant viral particles in the preparation by integrating and summing and the area under all the peaks of the plot.

[0049] In some embodiments, the invention provides methods of monitoring the removal of empty capsids and/or recombinant viral particle variants during the purification of a

preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) by using AUC. Samples of the recombinant viral particles from the preparation following one or more steps in the purification process are analyzed for the relative amount of empty capsids and/or recombinant viral particle variants wherein a decrease in the relative amount of empty capsids and/or recombinant viral particle variants to full capsid particles indicates removal of empty capsids and/or recombinant viral particle variants from the preparation of recombinant viral particles.

[0050] In some embodiments, the invention provides methods of evaluating processes for the production of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) by AUC. The preparation of recombinant viral particles is analyzed for the presence of intact full viral capsid particles, empty particles and/or recombinant viral particle variants. An increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral particle variants (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates, and the like) compared to a reference preparation of recombinant viral particles (*e.g.*, a standard recombinant viral preparation process) indicates an improvement in the production of recombinant viral particles.

I. General Techniques

[0051] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, *et al.* eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual (*Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998);

Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Ausubel *et al.*, eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway *et al.*, 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita *et al.*, eds., J.B. Lippincott Company, 2011).

II. Definitions

[0052] A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

[0053] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate (P-NH_2) or a mixed phosphoramidate- phosphodiester oligomer. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under

appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0054] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0055] A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs).

[0056] A "recombinant AAV vector (recombinant adeno-associated viral vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one AAV inverted terminal repeat sequences (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (*i.e.* AAV Rep and Cap proteins). When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral

vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an AAV particle. A recombinant viral vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (recombinant viral particle)".

[0057] A "recombinant adenoviral vector" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of adenovirus origin) that are flanked by at least one adenovirus inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that is expressing essential adenovirus genes deleted from the recombinant viral genome (*e.g.*, E1 genes, E2 genes, E4 genes, etc.). When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.,* in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of adenovirus packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an adenovirus particle. A recombinant viral vector can be packaged into an adenovirus virus capsid to generate a "recombinant adenoviral particle."

[0058] A "recombinant lentivirus vector" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentivirus origin) that are flanked by at least one lentivirus terminal repeat sequences (LTRs). In some embodiments, the recombinant nucleic acid is flanked by two lentiviral terminal repeat sequences (LTRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. A recombinant

lentiviral vector can be packaged into a lentivirus capsid to generate a "recombinant lentiviral particle."

[0059] A "recombinant herpes simplex vector (recombinant HSV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of HSV origin) that are flanked by HSV terminal repeat sequences. Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of HSV packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an HSV particle. A recombinant viral vector can be packaged into an HSV capsid to generate a "recombinant herpes simplex viral particle."

[0060] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (*e.g.*, a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0061] The term "transgene" refers to a polynucleotide that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

[0062] The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant viral DNA genome or RNA genome, regardless of infectivity or functionality. The number of

genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark *et al.* (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk *et al.* (2002) *Mol. Ther.*, 6:272-278.

[0063] The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant viral vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example with AAV, in McLaughlin *et al.* (1988) *J. Virol.*, 62:1963-1973.

[0064] The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant viral vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example regarding AAV, in Xiao *et al.* (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher *et al.* (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0065] An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0066] An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0067] A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins.

[0068] A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A helper virus provides "helper

functions" which allow for the replication of AAV. A number of such helper viruses have been identified, including adenoviruses, herpesviruses and poxviruses such as vaccinia. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Examples of adenovirus helper functions for the replication of AAV include E1A functions, E1B functions, E2A functions, VA functions and E4orf6 functions.

[0069] A preparation of rAAV is said to be "substantially free" of helper virus if the ratio of infectious AAV particles to infectious helper virus particles is at least about 10^2:l; at least about 10^4:l, at least about 10^6:l; or at least about 10^8:l or more. In some embodiments, preparations are also free of equivalent amounts of helper virus proteins (*i.e.*, proteins as would be present as a result of such a level of helper virus if the helper virus particle impurities noted above were present in disrupted form). Viral and/or cellular protein contamination can generally be observed as the presence of Coomassie staining bands on SDS gels (*e.g.*, the appearance of bands other than those corresponding to the AAV capsid proteins VPl, VP2 and VP3).

[0070] As used herein, "differential coefficient distribution value" or "C(S)" is a variant of the distribution of Lamm equation solutions to describe distributions of sedimenting particles; for example during ultracentrifugation.

[0071] As used herein, "Svedberg units" refers to a unit for sedimentation rate. The sedimentation rate for a particle of a given size and shape measures how fast the particle sediments. One Svedberg unit is equivalent to 10^{-13} seconds. For example, Svedberg units are often used to reflect the rate at which a molecule travels under the centrifugal force of a centrifuge.

[0072] As used herein, "sedimentation velocity conditions" or "boundary sedimentation velocity conditions" may refer to any experimental conditions under which a sample solution is

subjected to sedimentation velocity analysis. Sedimentation velocity allows the study of particles over a wide range of pH and ionic strength conditions and at temperatures 4 to 40 °C. The rate at which the sedimentation boundary moves is a measure of the sedimentation coefficient of the sedimenting species. The sedimentation coefficient depends on the molecular weight (larger particles sediment faster) and also on molecular shape. The minimum width of the sedimentation boundary is related to the diffusion coefficient of the molecule; the presence of multiple species with similar sedimentation coefficients will cause the boundary to be broader than expected on the basis of diffusion alone. Sedimentation velocity conditions may include without limitation any conditions related to the rotor speed, distance between sample and rotor center, temperature, solvent, sample, buffer, ultracentrifugation time, time interval for detection, sector and optical window characteristics, AUC instrumentation (including ultracentrifuge and detection apparatus), equilibrium dialysis of reference solvent, and data analysis algorithms.

[0073] As used herein, the term "analytical density gradient sedimentation equilibrium" relates to methods for measuring the buoyant density of a particle, or using differences in buoyant density to separate different species of particles. These methods may use, for example, AUC sedimentation equilibrium techniques. In these methods, a particle solution (*e.g.,* without limitation, a solution of a polypeptide, polynucleotide, or viral capsids) may be subjected to ultracentrifugation in a gradient solvate, such as a cesium chloride or cesium sulfate gradient, until equilibrium with the solvate is attained. At equilibrium, the particle solution will concentrate, or band, at the position in the gradient where the density of the particle is equal to that of the solvate. The position of bands may be used to calculate particle density, or a band may be extracted to isolate a single species of particle.

[0074] As used herein, the "SEDFIT algorithm" is an algorithm that allows one to analyze hydrodynamic data such as sedimentation velocity (Schuck (2000) *Biophys. J.*, 78:1606-19). In the SEDFIT algorithm, a grid of sedimentation coefficients across an expected range is created. Sedimentation boundaries are simulated using solutions to the Lamm equation for each sedimentation coefficient, assuming constant particle shape and solvent frictional ratio.

[0075] As used herein, the term "F statistic" or "F ratio" refers to the confidence level. This parameter controls the amount of regularization used. It has a different meaning for different ranges: From 0 to 0.5, no regularization is used. Values from 0.5 to 0.999 correspond to probabilities P (confidence levels). From these P-values, the desired chi-square increase allowed for the parsimony constraint of the regularization is calculated with F-statistics. A value of 0.51 will cause very little regularization; values of 0.68 to 0.90 would correspond to commonly used confidence levels (usually, with 50 scans or more the chi-square increase corresponding to a probability of 0.7 is of the order of 0.1%), while values close to 0.99 would cause very high regularization. The relationship of these values with probabilities can be examined using the F-statistics calculator. If numbers > 1 are entered, they are taken directly as chi-square ratios (as there are no probabilities > 1). For example, a value of 1.1 will result in regularization with 10% chi-square increase.

[0076] To "reduce" is to decrease, reduce or arrest an activity, function, and/or amount as compared to a reference. In certain embodiments, by "reduce" is meant the ability to cause an overall decrease of 20% or greater. In another embodiment, by "reduce" is meant the ability to cause an overall decrease of 50% or greater. In yet another embodiment, by "reduce" is meant the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater.

[0077] A "reference" as used herein, refers to any sample, standard, or level that is used for comparison purposes. For example, when measuring absorbance or refraction of AAV in an aqueous solution, the absorbance or refraction of the solution is compared to the absorbance or refraction of the aqueous solution without AAV (*i.e.* a reference solution). In other examples, a reference may refer to a standard procedure known in the art. For example, when analyzing a procedure for improved quality of AAV production (*e.g.,* homogeneity), the AAV produced by the candidate procedure is compared to procedures known in the art (*i.e.* reference procedures).

[0078] An "isolated" molecule (*e.g.*, nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment. Thus, for example, isolated rAAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of

DNase-resistant particles (DRPs) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like, as defined below.

[0079] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X."

[0080] As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise. For example, the phrase "a rAAV particle" includes one or more rAAV particles.

[0081] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Analytical ultracentrifugation

[0082] Analytical ultracentrifugation is a means to evaluate the molecular weight and the hydrodynamic and thermodynamic properties of a protein or other macromolecule. Heterogeneity of a protein or macromolecule by sedimentation velocity over a range of conditions including concentration, temperature, ionic strength, and pH. For example, a protein may be analyzed in a clinically relevant formulation. Use of analytical ultracentrifugation to characterize adenovirus preparations is provided by Berkowitz, SA & Philo JS, (2007) *Anal. Biochem.*, 362:16-37.

[0083] In certain aspects, the present invention provides methods of characterizing preparations of viral particles using analytical ultracentrifugation (AUC). For example, in some embodiments, the invention provides methods to assess vector genome integrity of recombinant adeno-associated viral (rAAV) particles in preparations of rAAV particles using AUC to distinguish viral particles with full, intact genomes, empty viral capsids and viral particles with variant (*e.g.,* truncated, aggregates, impurities and the like) viral genomes. In others embodiments, these methods may be applied in a similar way to analyze adenovirus,

lentivirus, and herpes simplex virus (HSV) particles. AUC analysis refers to quantitative methods for characterizing the biophysical properties of particles (*e.g.*, polypeptides, polynucleotides, and viral capsids) by measuring their migration through a solvent in a centrifugal field. AUC analysis has been well characterized over many decades and is highly versatile. Because AUC analysis relies upon first-principle hydrodynamic and thermodynamic information, AUC may be applied to determine the biophysical properties of many types of particles across a wide range of particle concentrations and sizes. AUC analysis typically encompasses two basic types of experiment: sedimentation velocity and sedimentation equilibrium. Sedimentation equilibrium analysis yields thermodynamic properties of particles that may be used to measure characteristics such as stoichiometry and association constants. Sedimentation velocity yields hydrodynamic properties of particles that may be used to measure characteristics such as size, shape, and concentration. A feature of AUC analysis of viral preparations is that the same assay conditions may be used to analyze different preparations of viral particles regardless of nucleotide sequence of the viral genome or serotype of the capsid.

[0084] Certain aspects of the present disclosure relate to the use of sedimentation velocity analysis to characterize viral capsid properties. In some embodiments, sedimentation velocity analysis uses an ultracentrifuge velocity cell with two sectors in dialysis equilibrium (one for an experimental sample and one for a solvent-only reference sample), each containing two optical windows that allow light to pass through the compartment. Ultracentrifugation applies an angular velocity to the cell and leads to rapid sedimentation of the solute particles towards the bottom of the sector. As sedimentation occurs, solute is depleted near the meniscus at the top of the cell, creating a sedimenting boundary between the depleted region and the sedimenting solute. The rate of movement or migration of the sedimenting boundary is measured by taking measurements that compare the properties of the sample and reference sectors at specific time intervals (for sedimentation velocity, these intervals are typically on the order of minutes). If multiple species of solute are present, this may lead to the formation of multiple sedimenting boundaries, each corresponding to a resolvable species.

[0085] Several methods for optically detecting a sedimenting boundary and measuring its rate of movement or migration are known in the art (for reference, see Cole *et al.* (2008)

Methods Cell Biol., 84:143-79). In some embodiments, the reference and sample sectors may be assayed using absorbance detection. In this detection method, the absorbance at a particular wavelength may be measured for the sample and reference sectors at different radial positions within each sector. Alternatively, the time course of absorbance at a single radial position may be measured. Beer's Law provides a mathematical relationship between absorbance and a solute's extinction coefficient.

[0086] In some embodiments, the reference and sample sectors may be assayed using interference detection (*e.g.*, Rayleigh interference detection). In the Rayleigh interference detection method, the interference optical system contains two parallel slits. A single, coherent beam of light is split such that it passes through both windows, and then the two beams are re-merged. When these two light waves are merged, they form an interference pattern of alternating light and dark fringes. If the sample and reference samples were to have an identical refractive index, the resulting interference fringes would be perfectly straight. Increasing the concentration of solute increases the solution's refractive index, thereby retarding the sample light beam and causing a vertical fringe shift. By measuring this fringe shift, one may measure the concentration of solute in the sample. Unlike absorbance detection, which measures absolute values for the sample and reference, interference detection measures a relative difference between the sample and reference. However, interference detection yields integrated peaks that are directly proportional to concentration, and it may be used for types of samples that do not absorb significantly. For a reference on using Rayleigh interference optics with AUC, see Furst (1997) *Eur. Biophys. J.* 35:307-10.

[0087] Measurement of the rate at which the sedimentation boundary moves may be used to derive many physical properties of solute particles. The rate of the boundary movement determines the sedimentation coefficient, which is based on the mass and shape (frictional coefficient) of the particle. The sedimentation coefficient of a particle, *s*, refers to the ratio of its velocity to the acceleration applied to it by a centrifugal field. Sedimentation coefficients are expressed in Svedberg units, S (one Svedberg unit is equivalent to 10^{-13} seconds). The sedimentation coefficient of a particle or solution of particles depends upon its properties, for example molecular weight (corrected for buoyancy), and the properties of the solvent.

[0088] The change in the concentration boundary of a solute over time during ultracentrifugation may be determined using the Lamm equation (Schuck (2000) *Biophys. J.*, 78:1606-19). Briefly, the Lamm equation calculates the change in the concentration boundary of a solute over time in response to the competing forces of sedimentation (which concentrates the solute) and diffusion (which disperses the solute), taking into account the sector-shaped cell and the centrifugal field generated by the rotor. The Lamm equation may be expressed as:

Equation 1: $\partial c/\partial t = D\,[(\partial^2 c/\partial r^2) + 1/r(\partial c/\partial r)] - s\omega^2\,[r(\partial c/\partial r) + 2c]$

where c is the solute concentration, D represents the solute diffusion constant, s represents the sedimentation coefficient, ω represents the angular velocity of the rotor, r is the radius, and t is time.

[0089] By fitting raw AUC data to solutions of the Lamm equation, it is possible to determine solute characteristics such as the sedimentation coefficient and the change in concentration distribution. For example, experimentally determined values for the rate of change of a sedimenting boundary may be modeled using the Lamm equation to derive the sedimentation coefficient, molecular mass, or concentration of the solute forming the boundary. Several programs known in the art, such as SEDFIT (Schuck (2000) *Biophys. J.*, 78:1606-19), may be used to model the Lamm equation to AUC data. These programs are also able to apply the Lamm equation to solutions containing multiple solutes or multiple sedimenting boundaries.

[0090] One example of a suitable program for the determination of solute characteristics is the SEDFIT algorithm. In some embodiments, the SEDFIT algorithm may be used to calculate a differential coefficient distribution value, or C(S), using AUC data from a solution containing a mixture of particle species (for reference, see Schuck (2000) *Biophys. J.,* 78:1606-19). In the SEDFIT algorithm, a grid of sedimentation coefficients across an expected range is created. Sedimentation boundaries are simulated using solutions to the Lamm equation for each sedimentation coefficient, assuming constant particle shape and solvent frictional ratio. Actual AUC data are then fit to these Lamm solutions to derive the differential coefficient distribution value, or C(S). Many other programs useful for analyzing AUC data may be found in Cole and Hansen (1999) *J. Biomol. Tech.* 10:163-76.

[0091] In some embodiments of the invention, recombinant viral particles are highly purified, suitably buffered, and concentrated. In some embodiments, the viral particles are concentrated to at least about any of 1 x 10^7 vg/mL, 2 x 10^7 vg/mL, 3 x 10^7 vg/mL, 4 x 10^7 vg/mL, 5 x 10^7 vg/mL, 6 x 10^7 vg/mL, 7 x 10^7 vg/mL, 8 x 10^7 vg/mL, 9 x 10^7 vg/mL, 1 x 10^8 vg/mL, 2 x 10^8 vg/mL, 3 x 10^8 vg/mL, 4 x 10^8 vg/mL, 5 x 10^8 vg/mL, 6 x 10^8 vg/mL, 7 x 10^8 vg/mL, 8 x 10^8 vg/mL, 9 x 10^8 vg/mL, 1 x 10^9 vg/mL, 2 x 10^9 vg/mL, 3 x 10^9 vg/mL, 4 x 10^9 vg/mL, 5 x 10^9 vg/mL, 6 x 10^9 vg/mL, 7 x 10^9 vg/mL, 8 x 10^9 vg/mL, 9 x 10^9 vg/mL, 1 x 10^{10} vg/mL, 2 x 10^{10} vg/mL, 3 x 10^{10} vg/mL, 4 x 10^{10} vg/mL, 5 x 10^{10} vg/mL, 6 x 10^{10} vg/mL, 7 x 10^{10} vg/mL, 8 x 10^{10} vg/mL, 9 x 10^{10} vg/mL, 1 x 10^{11} vg/mL, 2 x 10^{11} vg/mL, 3 x 10^{11} vg/mL, 4 x 10^{11} vg/mL, 5 x 10^{11} vg/mL, 6 x 10^{11} vg/mL, 7 x 10^{11} vg/mL, 8 x 10^{11} vg/mL, 9 x 10^{11} vg/mL, 1 x 10^{12} vg/mL, 2 x 10^{12} vg/mL, 3 x 10^{12} vg/mL, 4 x 10^{12} vg/mL, 5 x 10^{12} vg/mL, 6 x 10^{12} vg/mL, 7 x 10^{12} vg/mL, 8 x 10^{12} vg/mL, 9 x 10^{12} vg/mL, 1 x 10^{13} vg/mL, 2 x 10^{13} vg/mL, 3 x 10^{13} vg/mL, 4 x 10^{13} vg/mL, 5 x 10^{13} vg/mL, 6 x 10^{13} vg/mL, 7 x 10^{13} vg/mL, 8 x 10^{13} vg/mL, 9 x 10^{13} vg/mL. In some embodiments, the viral particles are concentrated to of about 1 x 10^7 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{11} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{12} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^{11} vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^9 vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^9 vg/mL, or about 1 x 10^7 vg/mL to about 1 x 10^8 vg/mL.

[0092] In some embodiments, viral particles are generated in a suitable host cells and purified. In some embodiments, the viral particles are purified by affinity chromatography. Methods to purify viral particles (*e.g.*, AAV particles, adenovirus particles, lentivirus particles, HSV particles) are known in the art. For example, by use of an antibody of a viral capsid protein or binding ligand of a viral capsid protein immobilized on a chromatography media. Examples of viral capsid affinity chromatographies include but are not limited to AVB affinity

chromatography for AAV (GE Healthcare), metal affinity chromatography for adenovirus and HSV, and heparin affinity chromatography for AAV and lentivirus, and the like. Methods to purify adenovirus particles are found, for example, in Bo, H *et al.*, (2014) *Eur. J. Pharm. Sci.* 67C:119-125. Methods to purify lentivirus particles are found, for example, in Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for example, in Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79.

[0093] In some embodiments, the recombinant viral particles are formulated in a pharmaceutical composition. In related embodiments, the pharmaceutical composition contains a buffer having physiological pH and/or physiological osmolality. A nonlimiting example of a pharmaceutical formulation is phosphate buffered saline (PBS) and in some embodiments, the PBS can be at physiological osmolality (*e.g.*, about pH 7.2 and about 300 mOsm/L). In some embodiments, sample adjustments are made to target concentration by optical density measurement at 260nm from 0.1 to 1.0. In some examples, this concentration results in reproducible and consistent AUC data. In some examples, concentration of viral particles is adjusted either by direct dilution with PBS or further concentration; for example, by using a centrifugal filter device.

[0094] In some embodiments of the invention, sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis is performed using an analytical ultracentrifuge that is capable of characterizing a sample in its native state under biologically relevant solution conditions (*e.g.*, ProteomeLab™ XL-I (Beckman Coulter)). When using the ProteomeLab™ XL-1, sample is loaded into the sample sector of a two sector velocity cell, a vehicle control (*e.g.*, PBS without recombinant viral) is loaded into the corresponding reference sector. The sample is placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of about 20 °C and full vacuum are maintained for about one hour. In an exemplary embodiment, sedimentation velocity centrifugation is performed at about 20,000 RPM, about 20 °C, and about 0.003 cm radial step setting, with no delay and with no replicates. As noted below, different parameters may be used for centrifugation. In some embodiments, absorbance (260 nm) and/or interference optics (*e.g.*, Rayleigh interference optics) are used to simultaneously record radial concentration as a function of time until the

smallest sedimenting component clears the optical window. In some embodiments, the radial concentration is recorded until the sedimenting species with the lowest density clears the sector. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge. A sector may be a portion of an ultracentrifuge; for example an ultracentrifuge velocity cell. In some embodiments, a sector may be a portion of an ultracentrifuge where samples are detected. In some embodiments, the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell. In some embodiments, is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window. In some embodiments, the radial concentration is recorded for at least about any of 0.5 hours, 0.75 hours, 1.0 hours, 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, or 5.0 hours. In some embodiments, the radial concentration is recorded for between any of about 0.5 hours to about 0.75 hours, about 0.75 hours to about 1.0 hours, about 1.0 hours to about 1.5 hours, about 1.5 hours to about 2.0 hours, about 2 hours to about 3 hours, about 3 hours to about 4 hours, about 4 hours to about 5 hours. In some embodiments, the radial concentration is recorded for about 1.2 hours. Optimizing runs conditions may include, for example, continuing the run until all of the sedimenting species are fully sedimented to the bottom of the sector, with the temperature held constant at 20 °C and a speed between 18,000 rpm and 20,000 rpm. As noted below, other temperatures and speeds may be used.

[0095] The percent full capsid is determined by analyzing a multiple of scans (*e.g.*, 75) from each detection method using the SEDFIT continuous size C(S) distribution model. Second (2nd) derivative regularization is applied to the fitting. In some embodiments, the confidence level of F statistic is about 0.68. In some embodiments, the confidence level of F statistic is more than about any of 0.68, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95 or 0.99. In some embodiments, the confidence level of F statistic is about 0.68 to about 0.90. In some embodiments, the confidence level of F statistic is about 0.68 to about 0.99. In some embodiments, the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software. In some embodiments, the resolution is about any of 200S, 300S, 400S, 500S, 600S, 700S,

800S, 900S, or 1000S. In some embodiments, the resolution is between any of about 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, the resolution is about 200S. In some embodiments, the Smax is about any of 100S, 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the Smax is between any of about 100S to about 1000S, 100S to about 900S, 100S to about 800S, 100S to about 700S, 100S to about 600S, 100S to about 500S, 100S to about 400S, 100S to about 300S, 100S to about 200S, 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, Smax is about 200S to about 5000S. In some embodiments, wherein Smax is about 200S. In some embodiments, the frictional ratio is left to float to a value determined by centrifugation software. In some embodiments, the frictional ratio is about 1.0. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied. In some embodiments, the meniscus position is allowed to float, letting the software choose the optimal position. In some embodiments, the frictional ratio is allowed to float, letting the software choose the optimal position. The model fits the data to the Lamm equation, and the resulting size distribution is a "distribution of sedimentation coefficients" that looks like a chromatogram with the area under each peak proportional to

concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) are determined for each component in the distribution. In some embodiments, multiple AUC runs are independent assays, and each analysis the following attributes are monitored to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\ nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans.

[0096] In some embodiments of the invention, extinction coefficients are used to calculate molar concentration and the actual percent value of the intact vector peak from absorbance data. Molar absorbance extinction coefficients for both empty capsids ($C_{260}/_{capsid}$=3.72e6) and intact vector ($C_{260}/_{vector}$=3.00e7) can be calculated based on published formulae (Sommer *et al.* (2003) *Mol Ther.*, 7:122-8). Extinction coefficients are available for empty capsid and intact vector peaks. The C(S) values can be determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.,* 78:1606-19. Molar concentration of both intact vector and empty capsid can be calculated using Beer's Law and the percentage of full capsid are calculated from these values. In some embodiments, values are reported in terms of the percentage of full capsid.

[0097] In some embodiments, it is not possible to determine empirically the extinction coefficient of particular species of recombinant viral particles (*e.g.*, viral particles with fragmented genomes of unknown size and sequence). A relationship between S value and genome size may be established by analyzing recombinant viral vector preps with encapsidated viral genomes of known nucleotide size and a corresponding S value are determined as described herein. The calculated S values can be plotted to generate a standard curve to which recombinant viral species of unknown molecular weight or genome size can be compared to determine the molecular weight of the unknown species.

[0098] In some aspects, the invention provides methods of characterizing a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at

time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle. In some embodiments, the species of recombinant viral particle identified by the methods of the invention include, but are not limited to; full recombinant viral particles comprising intact recombinant viral genomes, empty recombinant viral capsid particles, and recombinant viral particles comprising variant recombinant viral genomes. In some embodiments the variant genomes are smaller than the intact recombinant viral genome (*e.g.*, truncated genomes). In some embodiments, the variant genomes are larger than the intact recombinant viral genome (*e.g.,* aggregates, recombinants, *etc.*). In some embodiments, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of different known size. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles. In some embodiments, the sedimentation of recombinant viral particles is monitored at one time interval. In some embodiments, the sedimentation of recombinant viral particles is monitored at more than one time interval.

[0099] In some embodiments of the invention, the sedimentation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) is monitored by measuring optical density or absorbance at about 260 nm. Means of measuring absorbance are known in the art. In some embodiments, an ultracentrifuge used for AUC is equipped with means for measuring absorbance. In other embodiments, the sedimentation of recombinant viral particles is

monitored by interference. In some embodiments, the sedimentation of recombinant viral particles is monitored by Rayleigh interference. Means of measuring interference are known in the art (Furst (1997) *Eur. Biophys. J.* 35:307-10). In some embodiments, an ultracentrifuge used for AUC is equipped with means for measuring interference. In some embodiments, the sedimentation of recombinant viral particles is monitored by both absorbance and interference. In some embodiments, the absorbance and/or interference are measured using a reference standard. In some embodiments, the reference standard matches the solution of the recombinant viral preparation with the exception that the recombinant viral is not present. For example, the recombinant viral preparation may comprise recombinant viral in a buffer such as phosphate buffered saline. In this example, the reference standard may be phosphate buffered saline without recombinant viral particles.

[0100] In some embodiments of the invention, the preparation of viral particles is in a pharmaceutical formulation. Such formulations are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580). Such pharmaceutical formulations can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical formulation may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. In some embodiments of the invention the pharmaceutical formulation comprises phosphate buffered saline.

[0101] In some embodiments of the invention, the sedimentation velocity of viral particles during ultracentrifugation is determined by monitoring the sedimentation of viral particles continuously during ultracentrifugation. It is within the purview of the skilled artisan to optimize the parameters of AUC for different types of viral particles. Without wishing to be bound to theory, a range of AUC settings that allows the analysis of both AAV and adenovirus particles should enable the analysis of other viral particles including lentivirus and HSV since the size of HSV and lentiviral particles is between that of AAV and adenovirus particles. In some embodiments, data acquisition for rAAV, rHSV, lentiviral, and/or rAd particles is

performed with an AUC speed of between about 3,000 and about 20,000 rpm. In some embodiments, data analysis for rAAV, HSV, lentiviral, and/or adenoviral particles is performed with an S_{min} of about 1S and an S_{max} of about 1000S. In some embodiments, data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with a resolution of about 200S to about 1,000S. In some embodiments, the resolution is about any of 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the resolution is between any of about 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, the resolution is about 200S. data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with an Smax of about any of 100S, 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the Smax is between any of about 100S to about 1000S, 100S to about 900S, 100S to about 800S, 100S to about 700S, 100S to about 600S, 100S to about 500S, 100S to about 400S, 100S to about 300S, 100S to about 200S, 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, Smax is about 200S to about 5000S. In some embodiments, wherein Smax is about 200S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied. In some embodiments, the meniscus position is allowed to float,

letting the software choose the optimal position. In some embodiments, the frictional ratio is allowed to float, letting the software choose the optimal position. In some embodiments, data analysis for rAAV and/or adenoviral particles is held constant at 1. In some embodiments, data analysis for rAAV, HSV, lentiviral, and/or adenoviral particles is allowed to float by using the FIT command with a value optimized using non-linear regression.

[0102] With respect to recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles), in some embodiments, the sedimentation velocity of recombinant viral during ultracentrifugation is determined by monitoring (*e.g.*, scanning) the sedimentation of recombinant viral particles once in more than about every 15 seconds, 30 seconds, 45 seconds, 1 minute (60 seconds), 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes. Scans may be continuously acquired without delay as quickly as the optical systems allow. Interference scans are rapid, and a single scan is complete in ~10-15 seconds, while absorbance scans require ~ 60 seconds. When dual detection is used the speed of scan acquisition for both are determined by the absorbance system. In some embodiments of the invention, more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 scans are used to monitor sedimentation of recombinant viral particles during ultracentrifugation. In some embodiments, a minimum of 30 scans is required for analysis, and scans are collected until the sedimentation process is complete. In some embodiments, the sedimentation process may typically be described by between 40 and 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 55 scans to about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 55 scans to about 60 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 60 scans to about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 60 scans to about 70 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on multiple ultracentrifugations (runs). In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more ultracentrifugation runs. In some embodiments, the sedimentation velocities are used to

determine C(S) values using the SEDFIT algorithm. In some embodiments, a second derivative regularization is applied to a fitting level with a confidence level of F statistic of about 0.68. In some embodiments, the following C(S) parameters are held constant: resolution 100S to about 200S, S min is about 1, S max is about 200S to 300S, and frictional ratio is about 1.0 to 1.2S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

[0103] In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at more than about any of 5,000 rpm; 10,000 rpm; 15,000 rpm; 20,000 rpm; 25,000 rpm; 30,000 rpm; 35,000 rpm; 40,000 rpm; 45,000 rpm; or 50,000 rpm. In some embodiments, the ultracentrifugation is run at between any of about 5,000 rpm and about 50,000 rpm; about 10,000 rpm and about 50,000 rpm; about 15,000 rpm and about 50,000 rpm; about 20,000 rpm and about 50,000 rpm; about 25,000 rpm and about 50,000 rpm; about 30,000 rpm and about 50,000 rpm; about 35,000 rpm and about 50,000 rpm; about 40,000 rpm and about 50,000 rpm; about 45,000 rpm and about 50,000 rpm; about 5,000 rpm and about 45,000 rpm; about 10,000 rpm and about 45,000 rpm; about 15,000 rpm and about 45,000 rpm; about 20,000 rpm and about 45,000 rpm; about 25,000 rpm and about 45,000 rpm; about 30,000 rpm and about 45,000 rpm; about 40,000 rpm and about 45,000 rpm; about 5,000 rpm and about 40,000 rpm; about 10,000 rpm and about 40,000 rpm; about 15,000 rpm and about 40,000 rpm; about 20,000 rpm and about 40,000 rpm; about 25,000 rpm and about 40,000 rpm; about 30,000 rpm and about 40,000 rpm; about 35,000 rpm and about 40,000 rpm; about 5,000 rpm and about 35,000 rpm; about 10,000 rpm and about 35,000 rpm; about 15,000 rpm and about 35,000 rpm; about 20,000 rpm and about 35,000 rpm; about 25,000 rpm and about 35,000 rpm; about 30,000 rpm and about 35,000 rpm; about 5,000 rpm and about 30,000 rpm; about 10,000 rpm and about 30,000 rpm; about 15,000 rpm and about 30,000 rpm; about 20,000 rpm and about 30,000 rpm; about 25,000 rpm and about 30,000 rpm; about 5,000 rpm and about 25,000 rpm; about 10,000 rpm and about 25,000 rpm; about 20,000 rpm and about 25,000 rpm; about 5,000 rpm and about 20,000 rpm; about 10,000 rpm and about 20,000 rpm; about 15,000 rpm and about 20,000 rpm; about 5,000 rpm and about 15,000 rpm; about 10,000 rpm and about 15,000 rpm; or about 5,000 rpm and about 10,000 rpm. In some embodiments of the invention, the

boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 20,000 rpm. In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 15,000 rpm to about 20,000 rpm.

[0104] In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) by ultracentrifuging the preparation of recombinant viral particles at about or more than 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, or 30 °C. In some embodiments, the ultracentrifugation is run as between any of about 4 °C and about 30 °C, about 4 °C and about 25 °C, about 4 °C and about 20 °C, about 4 °C and about 15 °C, about 4 °C and about 10 °C, about 10 °C and about 30 °C, about 10 °C and about 25 °C, about 10 °C and about 20 °C, about 10 °C and about 15 °C, about 15 °C and about 30 °C, about 15 °C and about 25 °C, about 15 °C and about 20 °C, about 20 °C and about 30 °C, or about 20 °C and about 25 °C. In some embodiments, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 20 °C. In some embodiments, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 15 °C to about 20 °C.

[0105] As disclosed herein, numerous types of recombinant viral particles may be analyzed by the methods of the present disclosure (*e.g.,* AAV, adenoviral, lentiviral, and/or HSV particles). Suitable ultracentrifugation conditions, analysis algorithms, and other parameters may be determined empirically through methods known in the art. Exemplary parameters for AAV, adenoviral, lentiviral, and HSV particles, along with guidance for the selection of specific parameter options, are provided without limitation in Table 1 below.

Table 1. Exemplary parameters for AAV, adenoviral, lentiviral, and HSV particles.

	AAV	Ad	Lentivirus	HSV
Exemplary buffers	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L
Exemplary algorithms for determining C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)
Exemplary number of scans (minimum, maximum, ranges)	30-999 *excess scans can always be collected and then excluded from analysis (such as skip every other scan) -scans that occur after complete sedimentation of virus can be excluded	30-999	30-999	30-999
Exemplary confidence level of the F statistic	F=0.68	F=0.68	F=0.68	F=0.68
Exemplary ranges for Smin	1-100S	1-100S	1-100S	1-100S
Exemplary ranges for Smax	100-1000S	100-5000S	100-5000S	100-5000S
Exemplary ranges for resolution	*Resolution depends on S Max 200S-1000S	200-5000S	200-5000S	200-5000S
Exemplary frictional ratio	Use the FIT command to determine frictional ratio. Since AAV is ~spherical, in embodiments, 1 may be used as the frictional ratio.	Use FIT command to determine frictional ratio or set at 1.	Use FIT command to determine frictional ratio	Use FIT command to determine frictional ratio

Exemplary ranges for AUC speed	10,000-20,000 rpm	3,000-10,000 rpm	3,000-10,000 rpm	3,000-10,000 rpm
Exemplary absorbances for monitoring sedimentation of viral particles	260nm 280 nm 230 nm	260nm 280 nm 230 nm	260nm 280 nm 230 nm	260nm 280 nm 230 nm
Exemplary methods for monitoring sedimentation of viral particles	IF Absorbance	IF Absorbance	IF Absorbance	IF Absorbance
Radial invariant (RI) and time invariant (TI) noise subtractions, alternative subtractions/calculations	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection
Scanning frequency	*when using absorbance detection system, limited by speed of absorbance scan (~60 Seconds)-scan as fast as system allows with no delay IF only: collect every 10 -60 seconds (10-60 second delay)	Scan with no delay through scan with 60 second delay	Scan with no delay through scan with 60 second delay	Scan with no delay through scan with 60 second delay
Temperature ranges	4°C -20°C	4°C -20°C	4°C -20°C	4°C -20°C

[0106] In some aspects, the invention provides methods to determine the presence of empty capsids in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peak that corresponds to the S value

of empty capsid particles indicates that presence of empty capsid particles. In some embodiments, the invention provides methods of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value corresponding to empty capsid particles is compared to the total amount of all recombinant viral particles in the preparation by integrating all peaks on the plot of C(S) vs. S.

[0107] In some aspects, the invention provides methods to determine the presence of recombinant viral particle variants in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peak that corresponds to the S value that differs from the S value of recombinant viral capsid particles comprising a full intact recombinant viral genome indicates that presence of recombinant viral particle variants. In some embodiments, the invention provides methods of measuring the relative amount recombinant viral particle variants in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the

amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value that differs from the S value of recombinant viral capsid particles comprising a full intact recombinant viral genome is compared to the total amount of all recombinant viral particles in the preparation by integrating all peaks on the plot of C(S) vs. S. In some embodiments, the recombinant viral particle variants comprise recombinant viral genomes that are smaller (*e.g.*, truncated) or larger than the full length intact viral genome. Other viral-encapsidated DNA impurities can also be detected.

[0108] In some embodiments, the invention provides methods of monitoring the removal of empty capsids and/or recombinant viral particles with variant genomes during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids using AUC as described herein. A decrease in the relative amount of empty capsids and/or recombinant viral particles comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

[0109] In some embodiments, the invention provides methods of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant viral particles in the preparation. In some embodiments, the species of recombinant viral particle identified by the methods of the invention include, but are not limited to full recombinant viral particles comprising intact recombinant viral genomes,

empty recombinant viral capsid particles, and recombinant viral particles comprising variant recombinant viral genomes. In some embodiments the variant genomes are smaller than the intact recombinant viral genome (*e.g.*, truncated genomes). In some embodiments, the variant genomes are larger than the intact recombinant viral genome (*e.g.*, aggregates, recombinants, *etc.*). In some embodiments the variant genomes include genomes that are smaller and larger than the intact recombinant viral genome.

[0110] In some embodiments, the invention provides methods of monitoring the heterogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the relative amount of full capsids comprising an intact recombinant viral genome, empty capsids and/or recombinant viral particles with variant genomes using AUC as described herein, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

[0111] In embodiments of the embodiments described above, the recombinant viral particles have been purified using one or more purification steps. Examples of purification steps include but are not limited to equilibrium centrifugation, anion exchange filtration, tangential flow filtration (TFF), apatite chromatography, heat inactivation of helper virus, hydrophobic interaction chromatography, immunoaffinity chromatography, size exclusion chromatography (SEC), nanofiltration, cation exchange chromatography, and anion exchange chromatography.

[0112] In embodiments of the embodiments described above, the recombinant viral particles comprise a self-complementary AAV (scAAV) genome. In some embodiments, the recombinant AAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, a therapeutic transgene coding strand) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the therapeutic transgene) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide

sequence along most or all of its length. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR. In some embodiments, the scAAV viral particles comprise a monomeric form of an scAAV genome. In some embodiments, the scAAV viral particles comprise the dimeric form of and scAAV genome. In some embodiments, AUC as described herein is used to detect the presence of rAAV particles comprising the monomeric form of an scAAV genome. In some embodiments, AUC as described herein is used to detect the presence of rAAV particles comprising the dimeric form of an scAAV genome. In some embodiments, the packaging of scAAV genomes into capsid is monitored by AUC described herein.

[0113] In embodiments of the embodiments described above, the rAAV particles comprise an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid (*e.g.*, a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid (*e.g.*, a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV DJ capsid (*e.g.*, an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In embodiments of the above embodiments described above, the rAAV particles comprise at least one AAV1 ITR, AAV2 ITR, AAV3 ITR, AAV4 ITR, AAV5 ITR, AAV6 ITR, AAV7 ITR, AAV8 ITR, AAVrh8 ITR, AAV9 ITR, AAV10 ITR, AAVrh10 ITR, AAV11 ITR, or AAV12 ITR. In some embodiments, the rAAV particles comprise ITRs from one AAV serotype and AAV capsid from another serotype. For example, the rAAV particles may comprise a therapeutic transgene flanked by at least one

AAV2 ITR encapsidated into an AAV9 capsid. Such combinations may be referred to as pseudotyped rAAV particles.

IV. Viral particles

[0114] The methods disclosed herein may find use, *inter alia,* in characterizing species of interest in a variety of viral particles (*e.g.,* viral particles with a full genome, as compared to viral particles with truncated genomes and/or viral particles comprising DNA impurities).

[0115] In some embodiments, the viral particle is a recombinant AAV particle comprising a nucleic acid comprising a transgene flanked by one or two ITRs. The nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises the protein coding sequence(s) of interest (*e.g.*, a therapeutic transgene) operatively linked components in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette. The expression cassette is flanked on the 5' and 3' end by at least one functional AAV ITR sequences. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson *et al.*, *PNAS*, 2000, 97(7)3428-32; Passini *et al.*, *J. Virol.*, 2003, 77(12):7034-40; and Pechan *et al.*, *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (*e.g.*, as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao *et al.*, *PNAS*, 2002, 99(18): 11854-6; Gao *et al.*, *PNAS*, 2003, 100(10):6081-6; and Bossis *et al.*, *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAV11, AAV12, a

tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid, or the like. In some embodiments, the nucleic acid in the AAV comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, AAV12 or the like. In further embodiments, the rAAV particle comprises capsid proteins of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAV11, AAV12 or the like. In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, *et al. J. Virol.* 2004, 78(12):6381).

[0116] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle can comprise AAV9 capsid proteins and at least one AAV2 ITR or it can comprise AAV2 capsid proteins and at least one AAV9 ITR. In yet another example, a rAAV particle can comprise capsid proteins from both AAV9 and AAV2, and further comprise at least one AAV2 ITR. Any combination of AAV serotypes for production of a rAAV particle is provided herein as if each combination had been expressly stated herein.

[0117] In some embodiments, the AAV comprises at least one AAV1 ITR and capsid protein from any of AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV2 ITR and capsid protein from any of AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV3 ITR and capsid protein from any of AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV4 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV5 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV6, AAV7, AAV8, AAV9, AAVrh.8,

AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV6 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV7 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV8 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV9 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAVrh8 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAVrh10 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV11 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAV9, AAVrh10, and/or AAV12. In some embodiments, the AAV comprises at least one AAV12 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV rh8, AAV9, AAVrh10, and/or AAV11.

Self-complementary AAV viral genomes

[0118] In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome. AAV viral particles with self-complementing genomes and methods of use of self-complementing AAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,765,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., *et al.*, (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (*e.g.*, complementing coding and non-coding strands of a transgene). In some embodiments, the invention provides an AAV viral particle comprising an AAV genome, wherein the rAAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, a therapeutic transgene

coding strand) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the therapeutic transgene) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide sequence along most or all of its length. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR (*e.g.*, the right ITR). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0119] In some embodiments, the viral particle is an adenoviral particle. In some embodiments, the adenoviral particle is a recombinant adenoviral particle, *e.g.,* a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of adenoviral origin) between two ITRs. In some embodiments, the adenoviral particle lacks or contains a defective copy of one or more E1 genes, which renders the adenovirus replication-defective. Adenoviruses include a linear, double-stranded DNA genome within a large (~950Å), non-enveloped icosahedral capsid. Adenoviruses have a large genome that can incorporate more than 30kb of heterologous sequence (*e.g.,* in place of the E1 and/or E3 region), making them uniquely suited for use with larger heterologous genes. They are also known to infect dividing and non-dividing cells and do not naturally integrate into the host genome (although hybrid variants may possess this ability). In some embodiments, the adenoviral vector may be a first generation adenoviral vector with a heterologous sequence in place of E1. In some embodiments, the adenoviral vector may be a second generation adenoviral vector with additional mutations or deletions in E2A, E2B, and/or E4. In some embodiments, the adenoviral vector may be a third generation or gutted adenoviral vector that lacks all viral coding genes, retaining only the ITRs and packaging signal and requiring a

helper adenovirus *in trans* for replication, and packaging. Adenoviral particles have been investigated for use as vectors for transient transfection of mammalian cells as well as gene therapy vectors. For further description, see, *e.g.,* Danthinne, X. and Imperiale, M.J. (2000) *Gene Ther.* 7:1707-14 and Tatsis, N. and Ertl, H.C. (2004) *Mol. Ther.* 10:616-29.

[0120] In some embodiments, the viral particle is a recombinant adenoviral particle comprising a nucleic acid comprising a transgene. Use of any adenovirus serotype is considered within the scope of the present invention. In some embodiments, the recombinant adenoviral vector is a vector derived from an adenovirus serotype, including without limitation, AdHu2, AdHu 3, AdHu4, AdHu5, AdHu7, AdHu11, AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, and porcine Ad type 3. The adenoviral particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise an adenoviral particle in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped recombinant adenoviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped recombinant adenoviral particles are derived from a foreign virus or from another adenovirus serotype. In some embodiments, the foreign viral capsid proteins are derived from, including without limitation, reovirus type 3. Examples of vector and capsid protein combinations used in pseudotyped adenovirus particles can be found in the following references (Tatsis, N. *et al.* (2004) *Mol. Ther.* 10(4):616-629 and Ahi, Y. *et al.* (2011) *Curr. Gene Ther.* 11(4):307-320). Different adenovirus serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes, include without limitation, lung (*e.g.* HuAd3), spleen and liver (*e.g.* HuAd37), smooth muscle, synoviocytes, dendritic cells, cardiovascular cells, tumor cell lines (*e.g.* HuAd11), and dendritic cells (*e.g.* HuAd5 pseudotyped with *reovirus type 3, HuAd30, or HuAd35*). For further description, see Ahi, Y. *et al.* (2011) *Curr. Gene Ther.* 11(4):307-320, Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40, and Tatsis, N. *et al.* (2004) *Mol. Ther.* 10(4):616-629.

[0121] In some embodiments, the viral particle is a lentiviral particle. In some embodiments, the lentiviral particle is a recombinant lentiviral particle, *e.g.,* a polynucleotide

vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentiviral origin) between two LTRs. Lentiviruses are positive-sense, ssRNA retroviruses with a genome of approximately 10 kb. Lentiviruses are known to integrate into the genome of dividing and non-dividing cells. Lentiviral particles may be produced, for example, by transfecting multiple plasmids (typically the lentiviral genome and the genes required for replication and/or packaging are separated to prevent viral replication) into a packaging cell line, which packages the modified lentiviral genome into lentiviral particles. In some embodiments, a lentiviral particle may refer to a first generation vector that lacks the envelope protein. In some embodiments, a lentiviral particle may refer to a second generation vector that lacks all genes except the gag/pol and tat/rev regions. In some embodiments, a lentiviral particle may refer to a third generation vector that only contains the endogenous rev, gag, and pol genes and has a chimeric LTR for transduction without the tat gene (see Dull, T. *et al.* (1998) *J. Virol.* 72:8463-71). For further description, see Durand, S. and Cimarelli, A. (2011) *Viruses* 3:132-59.

[0122] In some embodiments, the viral particle is a recombinant lentiviral particle comprising a nucleic acid comprising a transgene. Use of any lentiviral vector is considered within the scope of the present invention. In some embodiments, the lentiviral vector is derived from a lentivirus including, without limitation, human immunodeficiency virus-1 (HIV-1), human immunodeficiency virus-2 (HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), Jembrana disease virus (JDV), visna virus (VV), and caprine arthritis encephalitis virus (CAEV). The lentiviral particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise a lentivirus vector in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped recombinant lentiviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped recombinant lentiviral particles are derived from a foreign virus. In some embodiments, the foreign viral capsid protein used in pseudotyped recombinant lentiviral particles is Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped recombinant lentiviral particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped recombinant lentiviral particles. In other embodiments, the foreign viral capsid proteins are

derived from, including without limitation, Chandipura virus, Rabies virus, Mokola virus, Lymphocytic choriomeningitis virus (LCMV), Ross River virus (RRV), Sindbis virus, Semliki Forest virus (SFV), Venezuelan equine encephalitis virus, Ebola virus Reston, Ebola virus Zaire, Marburg virus, Lassa virus, Avian leukosis virus (ALV), Jaagsiekte sheep retrovirus (JSRV), Moloney Murine leukemia virus (MLV), Gibbon ape leukemia virus (GALV), Feline endogenous retrovirus (RD114), Human T-lymphotropic virus 1 (HTLV-1), Human foamy virus, Maedi-visna virus (MVV), SARS-CoV, Sendai virus, Respiratory syncytia virus (RSV), Human parainfluenza virus type 3, Hepatitis C virus (HCV), Influenza virus, Fowl plague virus (FPV), or Autographa californica multiple nucleopolyhedro virus (AcMNPV). Examples of vector and capsid protein combinations used in pseudotyped Lentivirus particles can be found, for example, in Cronin, J. *et al.* (2005). *Curr. Gene Ther.* 5(4):387-398. Different pseudotyped recombinant lentiviral particles can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). For example, tissues targeted by specific pseudotyped recombinant lentiviral particles, include without limitation, liver (*e.g.* pseudotyped with a VSV-G, LCMV, RRV, or SeV F protein), lung (*e.g.* pseudotyped with an Ebola, Marburg, SeV F and HN, or JSRV protein), pancreatic islet cells (*e.g.* pseudotyped with an LCMV protein), central nervous system (*e.g.* pseudotyped with a VSV-G, LCMV, Rabies, or Mokola protein), retina (*e.g.* pseudotyped with a VSV-G or Mokola protein), monocytes or muscle (*e.g.* pseudotyped with a Mokola or Ebola protein), hematopoietic system (*e.g.* pseudotyped with an RD114 or GALV protein), or cancer cells (*e.g.* pseudotyped with a GALV or LCMV protein). For further description, see Cronin, J. *et al.* (2005). *Curr. Gene Ther.* 5(4):387-398 and Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40.

[0123] In some embodiments, the viral particle is a herpes simplex virus (HSV) particle. In some embodiments, the HSV particle is a rHSV particle, *e.g.,* a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentiviral origin) between two TRs. HSV is an enveloped, double-stranded DNA virus with a genome of approximately 152 kb. Advantageously, approximately half of its genes are nonessential and may be deleted to accommodate heterologous sequence. HSV particles infect non-dividing cells. In addition, they naturally establish latency in neurons, travel by retrograde transport, and can be transferred across synapses, making them advantageous for transfection of neurons and/or gene therapy approaches involving the nervous system. In some embodiments, the HSV

particle may be replication-defective or replication-competent (*e.g.*, competent for a single replication cycle through inactivation of one or more late genes). For further description, see Manservigi, R. *et al.* (2010) *Open Virol. J.* 4:123-56.

[0124] In some embodiments, the viral particle is a rHSV particle comprising a nucleic acid comprising a transgene. Use of any HSV vector is considered within the scope of the present invention. In some embodiments, the HSV vector is derived from a HSV serotype, including without limitation, HSV-1 and HSV-2. The HSV particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise a HSV vector in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped rHSV particles. In some embodiments, foreign viral capsid proteins used in pseudotyped rHSV particles are derived from a foreign virus or from another HSV serotype. In some embodiments, the foreign viral capsid protein used in a pseudotyped rHSV particle is a Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped rHSV particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped rHSV particles. In other embodiments, the foreign viral capsid protein may be from a different HSV serotype. For example, an HSV-1 vector may contain one or more HSV-2 capsid proteins. Different HSV serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes include without limitation, central nervous system and neurons (*e.g.* HSV-1). For further description, see Manservigi, R. *et al.* (2010) *Open Virol J* 4:123-156, Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40, and Meignier, B. *et al.* (1987) *J. Infect. Dis.* 155(5):921-930.

V. Production of Viral Vectors

[0125] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids. rAAV production cultures for the production of rAAV virus particles all require; 1) suitable host cells, including, for example,

human-derived cell lines such as HeLa, A549, or 293 cells, or insect-derived cell lines such as SF-9, in the case of baculovirus production systems; 2) suitable helper virus function, provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus, baculovirus, or a plasmid construct providing helper functions; 3) AAV rep and cap genes and gene products; 4) a transgene (such as a therapeutic transgene) flanked by at least one AAV ITR sequences ; and 5) suitable media and media components to support rAAV production. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors.

[0126] Suitable rAAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, rAAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of rAAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of rAAV in production cultures.

[0127] In some aspects, the invention provides methods for preparing rAAV particles with reduced empty capsids comprising a) culturing host cells under conditions suitable for rAAV production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release rAAV particles; c) isolating the rAAV particles produced by the host cell; and d) analyzing the rAAV particles for the presence of empty capsids and/or rAAV particles with variant genomes by analytical ultracentrifugation as

described above. In some embodiments, the p5 promoter of the nucleic acid encoding AAV rep and cap regions is located 3' to the rep and/or cap coding region. In some embodiments, the nucleic acid encoding AAV rep and cap coding regions is plasmid pHLP, pHLP19, or pHLP09 (see US Patent Nos. 5,622,856; 6,001,650; 6,027,931; 6,365,403; 6,376,237; and 7,037,713; the content of each is incorporated herein in its entirety). In some embodiments, the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

[0128] rAAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, rAAV production cultures include attachment-dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. rAAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0129] rAAV vector particles of the invention may be harvested from rAAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of rAAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0130] Numerous methods are known in the art for production of adenoviral vector particles. For example, for a gutted adenoviral vector, the adenoviral vector genome and a helper adenovirus genome may be transfected into a packaging cell line (*e.g.,* a 293 cell line). In some embodiments, the helper adenovirus genome may contain recombination sites flanking its packaging signal, and both genomes may be transfected into a packaging cell line that expresses a recombinase (*e.g.,* the Cre/loxP system may be used), such that the adenoviral

vector of interest is packaged more efficiently than the helper adenovirus (see, *e.g.,* Alba, R. *et al.* (2005) *Gene Ther.* 12 Suppl 1:S18-27). Adenoviral vectors may be harvested and purified using standard methods, such as those described herein.

[0131] Numerous methods are known in the art for production of lentiviral vector particles. For example, for a third-generation lentiviral vector, a vector containing the lentiviral genome of interest with gag and pol genes may be co-transfected into a packaging cell line (*e.g.,* a 293 cell line) along with a vector containing a rev gene. The lentiviral genome of interest also contains a chimeric LTR that promotes transcription in the absence of Tat (see Dull, T. *et al.* (1998) *J. Virol.* 72:8463-71). Lentiviral vectors may be harvested and purified using methods (*e.g.*, Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011) described herein.

[0132] Numerous methods are known in the art for production of HSV particles. HSV vectors may be harvested and purified using standard methods, such as those described herein. For example, for a replication-defective HSV vector, an HSV genome of interest that lacks all of the immediate early (IE) genes may be transfected into a complementing cell line that provides genes required for virus production, such as ICP4, ICP27, and ICP0 (see, *e.g.,* Samaniego, L.A. *et al.* (1998) *J. Virol.* 72:3307-20). HSV vectors may be harvested and purified using methods described (*e.g.*, Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79).

VI. Purification of rAAV Vectors

[0133] At harvest, rAAV production cultures of the present invention may contain one or more of the following: (1) host cell proteins; (2) host cell DNA; (3) plasmid DNA; (4) helper virus; (5) helper virus proteins; (6) helper virus DNA; and (7) media components including, for example, serum proteins, amino acids, transferrins and other low molecular weight proteins. In addition, rAAV production cultures further include rAAV particles having an AAV capsid serotype selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAV9, AAV10, AAVrh10, AAV11, or AAV12. In some embodiments, the rAAV production cultures further comprise empty AAV capsids (*e.g.,* a rAAV particle comprising capsid proteins but no rAAV genome). In some embodiments, the rAAV production cultures further comprise rAAV particles comprising variant rAAV genomes

(*e.g.*, a rAAV particle comprising a rAAV genome that differs from an intact full-length rAAV genome). In some embodiments, the rAAV production cultures further comprise rAAV particles comprising truncated rAAV genomes. In some embodiments, the rAAV production cultures further comprise rAAV particles comprising AAV-encapsidated DNA impurities.

[0134] In some embodiments, the rAAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0135] In some embodiments, the rAAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0136] rAAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the rAAV particles; rAAV capture by apatite chromatography; heat inactivation of helper virus; rAAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and rAAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify rAAV particles are found, for example, in US Patent Numbers 6,989,264 and 8,137,948 and WO 2010/148143. Methods to purify adenovirus particles are found, for example, in Bo, H *et al.*, (2014) *Eur. J. Pharm. Sci.* 67C:119-125. Methods to purify lentivirus particles are found, for example, in Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for

example, in Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79.

EXAMPLES

[0137] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Characterization of recombinant adeno-associated viral vector preparations by analytical ultracentrifugation

[0138] Adeno-associated viruses (AAV) have features that make them attractive as vectors for gene therapy. Wild-type AAV consists of two open reading frames (rep and cap) which code for all structural and regulatory elements required for assembly, replication and infection. The rep ORF codes for Rep 78 and 68 proteins which have genome replication functions as well as Rep 52 and 40 proteins which are involved in single strand replication and packaging. The cap ORF codes for the three structural capsid proteins: VP1, VP2 and VP3. Recombinant AAV vector is typically produced by the triple transfection method using the "gutless" vector approach (Xiao, X, *et al.*, 1998, *J. Virol.* 3:2224-2232). The rep and cap genes are replaced with the therapeutic gene and its regulatory elements sandwiched between a 5' and 3' inverted terminal repeat (ITR), the rep and cap genes are provided in trans on a separate plasmid and a third plasmid contributes required adenoviral helper genes. It is postulated that the viral capsids are fully assembled and the ITR flanked vector genome is then inserted into the capsid via a capsid pore (Myers, MW & Carter, BJ, 1980, *Virology*, 102:71-82). The resulting population of capsids contains both non-genome containing capsids (empty capsids) as well as genome containing capsids. In addition, capsids may contain incomplete portions of the recombinant viral genome. The vector prep may then purified by affinity chromatography to isolate the capsids from the cellular debris and can be further processed to enrich for intact vector by anion exchange chromatography.

[0139] Based on their recent approval for use in gene therapy, adeno-associated viral (AAV) vectors have emerged as an important class of novel biopharmaceutical drug products. The generation of AAV vector products requires an analytical method that monitors product quality with regard to homogeneity, purity, and consistency of manufacturing, yet to date no method to support AAV vector characterization has been established. To meet this demand, the potential use of analytical ultracentrifugation (AUC) as a technique to characterize the homogeneity of AAV vectors was investigated.

Methods

Sample preparation

[0140] In order to support accurate AUC assessment, vector product (AAV2-transgene 2) was highly purified, suitably buffered, and concentrated to greater than 5 x 10^{11} vg/mL. To achieve this, cell supernatants runs were purified using AVB affinity chromatography (GE Healthcare) and buffer-exchanged into PBS, pH 7.2 using a 10K MWCO Slide-a-Lyzer (Thermo Scientific). Product concentration was determined by optical density measurement at 260nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260nm from 0.1 to 1.0, either by direct dilution with PBS or further concentration using Amicon Ultra-0.5 /30K MWCO Centrifugal Filter Device.

Sedimentation velocity AUC data acquisition

[0141] Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis was performed using a ProteomeLab™ XL-I (Beckman Coulter). 400 µL sample was loaded into the sample sector of a two sector velocity cell, and 400 µL PBS was loaded into the corresponding reference sector. The sample was placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of 20°C and full vacuum were maintained for one hour. Sedimentation velocity centrifugation was performed at 20,000 RPM, 20°C, 0.003 cm radial step setting, with no delay and with no replicates. Absorbance (260 nm) and Raleigh interference optics were used to simultaneously record radial concentration as a function of time until the smallest sedimenting component cleared the optical window (1.2 hour). Assay

throughput was limited to a single sample per run based on absorbance scan collection times of greater than one minute, as well as the large size and rapid sedimentation of AAV.

AUC data analysis

[0142] The percent full capsid was determined by analyzing approximately 75 scans from each detection method using the SEDFIT (NIH/see worldwide web at analyticalultracentrifugation.com) continuous size C(S) distribution model. Second (2nd) derivative regularization was applied to the fitting with a confidence level of F statistic=0.68. The following C(S) parameters were held constant: resolution=200S, S min=1, S max=200 and frictional ratio=1.0. RI and TI noise subtractions were applied, and the meniscus position was allowed to float, letting the software choose the optimal position. This model fit the data to the Lamm equation, and the resulting size distribution was a "distribution of sedimentation coefficients" that looked like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) were determined for each component in the distribution. Each AUC run was an independent assay, and each analysis was monitored for the following attributes to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\ nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans.

Absorbance optics (260 nm)

[0143] Extinction coefficients were used to calculate molar concentration and the actual percent value of the intact vector peak from absorbance data. Molar absorbance extinction coefficients for both empty capsids ($\epsilon_{260}/_{capsid}$=3.72e6) and intact vector ($\epsilon_{260}/_{vector}$=3.00e7) were calculated based on published formulae (Sommer *et al.* (2003) *Mol Ther.*, 7:122-8). Extinction coefficients were available for empty capsid and intact vector peaks. The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.*, 78:1606-19. Molar concentration of both intact vector and empty capsid were calculated using Beer's Law, and the percentage of full capsid was calculated from these values. Values were reported in terms of the percentage of full capsid.

Generation of an AUC standard curve

[0144] Because it is not possible to determine empirically the extinction coefficient of fragmented genomes of unknown size and sequence, a relationship between S value and genome size was established. To achieve this, rAAV vector preps with encapsidated viral genomes of known nucleotide size were analyzed by AUC, and a corresponding S value was determined as described above.

Production of rAAV by transient transfection

[0145] Recombinant AAV vector was produced by the triple transfection method using the "gutless" vector approach (Xiao *et al.* (1998) *J. Virol.*, 3:2224-32). In this approach, the rep and cap genes were replaced with the therapeutic gene and its regulatory elements, both sandwiched between a 5' and 3' inverted terminal repeat (ITR). The rep and cap genes were provided in trans on a separate plasmid, and a third plasmid contributed the required adenoviral helper genes. Without wishing to be bound to theory, it is postulated that the viral capsids are fully assembled, and the ITR flanked vector genome is then inserted into the capsid via a capsid pore (Myers & Carter (1980) *Virology*, 102:71-82). The resulting population of capsids contained both non-genome-containing capsids (empty capsids) and genome-containing capsids.

Production of rAAV by producer cell platform

[0146] The AAV producer cell line is an alternative production platform used to generate clinical rAAV vectors. With this method, a HeLa S3 cell, adapted to growth in suspension, was engineered to have integrated copies of the AAV rep and cap genes required for vector replication and packaging, in addition to vector sequences and the selectable marker (*see, e.g.*, Puro: Thorne *et al.* (2009) *Hum. Gene Ther.*, 20:707-14). Once infected with WT Adenovirus, which provides the helper functions required for replication, the cell produced recombinant AAV vector as well as adenovirus, which was subsequently removed during the purification process using ion-exchange chromatography.

Other methods

[0147] Synthetic transgenes were cloned into a plasmid that contained a promoter of choice and bovine growth hormone polyadenylation signal sequence (polyA). The entire transgene expression cassette was then cloned into previral plasmid vector pAAVDC64 containing AAV2 inverted terminal repeats. The total size of the resulting AAV genomes in the respective expression plasmids (including the region flanked by ITRs) was 4 - 4.6 kb. The recombinant vectors were produced by triple transfection of 293 cells using helper plasmids expressing rep2/cap sequences and Adenovirus helper functions, pAd Helper (Stratagene, La Jolla, CA USA) The rep/cap helper expressed rep from AAV serotype 2, while the cap sequence encoded one of the following sequences: AAV cap 1, 2, 5, 9, or rh8R. Vectors were purified by affinity chromatography and in some cases were further purified to remove empty particles (see, *e.g.*, Qu *et al.* (2007) J. *Virol. Methods.* 140:183-92).

Results

[0148] Analytical ultracentrifugation (AUC) using classical boundary sedimentation velocity was used to reveal the particle heterogeneities of recombinant adeno-associated virus (rAAV) vector preps. A mixture containing 20% rAAV2 particles with the full genome and 80% empty capsids was created by mixing together purified empty capsids and purified genome-containing capsids at defined ratios. The empty and full capsids were generated by $CsCl_2$ gradient purification of a mixture of empty and full capsids following triple transfection production. To monitor the movement of rAAV2 particles in response to a centrifugal force, this mixture of rAAV2 capsids was scanned at an absorbance of 260nm along a centrifugal field at defined time intervals. **FIG. 1A** shows a representative scanning profile following centrifugation of the AAV2 mixture at 20,000 rpm for 1.2 hrs (until the smallest sedimenting species cleared the optical window). Scans represented the acquisition of concentration data as a function of radius r, at times t, to yield a series of concentration scans that revealed the complete migration pattern of constituent vector particles in the rAAV2 vector prep. In these sigmoidal curves or boundaries, the leading edge of the curve represented the faster sedimenting species (*i.e.*, the genome-containing rAAV2 capsid), and the trailing edge of the

curve represented the slower sedimenting species (*i.e.*, the "empty" rAAV2 capsids) (**FIG. 1A**).

[0149] Plotting the differential sedimentation coefficient distribution value, C(S), versus the sedimentation coefficient (in Svedberg units, S) yielded distinct peaks with unique sedimentation coefficients for both the empty and genome-containing capsid species (**FIG. 1B**). The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.*, 78:1606-19. In order to calculate molar concentrations and percent value for each capsid species from the absorbance data, extinction coefficients were used according to Table 2.

Table 2. Extinction coefficients and molar concentrations for capsid species

Species	Signal (Abs $_{260nm}$)	ε_{260nm}	Molar concentration (M)	Relative abundance (%)
Peak 1	0.0479	3.73E+06	1.28E-08	79
Peak 2	0.0909	2.59E+07	3.51E-09	21
Sum			1.63E-08	

[0150] Molar absorbance extinction coefficients for both the empty capsid ($\varepsilon_{260}/_{capsid}$ = 3.72e6) and genome containing capsid ($\varepsilon_{260}/_{capsid}$ = 3e7) were calculated using genome size and published formulae (Sommer *et al.*, 2003). Molar concentrations of both genome-containing and empty capsids were then calculated using Beer's Law. The molar concentration of each species was used to calculate its relative abundance, expressed as a percentage of total capsids (**FIG. 1**). These results demonstrated that AUC may be used to accurately distinguish and quantify empty capsids and genome-containing capsids from a heterogeneous vector preparation.

[0151] For the rAAV2 vector prep shown in **FIG. 1**, capsids containing a full genome were represented by the peak sedimenting at 94S and accounted for 21% of the vector prep. Empty capsids sedimented with an S value of 64S and accounted for 79% of the vector prep. These sedimentation coefficient values were confirmed by AUC analysis of pure populations of empty (**FIG. 2A**) or genome containing particles (**FIG. 2B**). The AUC profile of a pure population of rAAV2 empty capsids revealed a single peak with a sedimentation coefficient of

64S, whereas the AUC profile of a pure population of rAAV2AUC genome-containing capsids revealed a single peak with a higher sedimentation coefficient of 94S. These results agreed with the values generated from a heterogeneous preparation and further confirmed that AUC methods may be used to quantify genome-containing and empty AAV capsids from a heterogeneous preparation containing both species.

[0152] The AUC method was further assessed for reproducibility by performing five independent AUC runs of the same vector sample (scAAV2/9 LP2), as shown in Table 3. The sedimentation coefficients for both genome-containing and empty AAV2 capsids were highly reproducible, yielding coefficients of variation from 0.5-0.6%. Similarly, the relative abundance (expressed as a percentage of the total) of genome-containing capsids was determined with a coefficient of variation of approximately 2%. These results indicated that the AUC method for quantifying genome-containing and empty AAV2 capsids yields highly reproducible and consistent values.

Table 3. Five independent assays on scAAV2/9 LP2 sample.

AUC Run	Empty Capsid, Peak 1 (S)	Full Capsid, Peak 3	
		% Full Capsids	S
20110927A	64.3	34.1	84.0
20111003A	64.6	34.2	84.6
20111005A	65.1	35.0	84.5
20111005B	64.2	35.2	83.8
20111011A	64.1	33.5	84.5
Mean	64.5	34.4	84.3
Standard Deviation	0.4	0.7	0.4
% CV	0.6	2.1	0.5

Example 2: Comparison of interference and absorbance detection methods for AUC

[0153] An alternative optical detection method for AUC, Rayleigh Interference Optics, was also evaluated. This detection method measures the sample concentration based on refractive index differences between a reference solution and the AAV containing sample. Like absorbance detection, interference detection can be applied to any rAAV regardless of the sequence of the genome. Unlike absorbance detection, which requires an extinction

coefficient, interference detection yields integrated peaks that are directly proportional to concentration.

[0154] Pure populations of empty and genome-containing AAV2 capsids were mixed at a 1:1 ratio and analyzed by AUC using both interference (**FIG. 3A**) and absorbance detection methods (**FIG. 3B**). Interference detection revealed two populations of AAV capsids at the approximate expected ratios of 43% empty and 57% genome-containing (**FIG. 3A**). Both detection methods yielded similar abundance ratios. However, comparing the peak sizes generated by both methods illustrates the disconnect between peak height and concentration with absorbance detection (compare size of "empty capsid" peaks in **FIG. 3A** and **3B**). The data generated by both methods are compared in Table 4. The ratio of absorbance signal to interference signal (A_{260nm}/IF) can be used in a fashion analogous to the 260/280 ratio of absorbance data, and this assisted in identifying peaks in the C(S) distribution.

Table 4. S values and relative abundance generated by absorbance and interference detection.

Peak	Absorbance (260 nm)		Interference			A_{260nm}/IF
	Sedimentation coefficient (S)	Relative abundance (%)	Sedimentation coefficient (S)	Signal (fringes)	Relative abundance (%)	
Peak 1	63	47	62	0.080	43	0.41
Peak 2	93	53	92	0.104	57	2.38
Sum				0.184		

[0155] Although interference optics offers precision and resolution, it may require a high concentration of sample. Moreover, interference optics may be affected by a mismatch between the reference and AAV sample buffer. AAV samples, however, typically contain a low protein concentration, and it may be necessary to completely match the AAV sample and reference buffers.

Example 3: Influence of production method on AAV vector heterogeneity

[0156] The previous examples demonstrated that the AUC method is a highly accurate and reproducible way to resolve and quantify empty and genome-containing AAV capsids from a heterogeneous mixture. This capability could be advantageous for a variety of applications to evaluate the quality of AAV vector preparations. For example, a major problem in producing

pure AAV vector preparations is the presence of capsids with partial or fragmented genomes. To illustrate the utility of the AUC method for resolving these species, AAV vectors generated by two different methods, termed the "triple transfection" and "producer cell line" methods, were analyzed by AUC.

[0157] An AAV2 vector harboring the transgene 2 was produced using either the triple transfection method (**FIG. 4**) or the producer cell line method (**FIG. 5**). For a description of these methods, see Example 1. Following chromatographic purification, both vector preps were analyzed by AUC. **FIG. 6A** shows a schematic of this AAV2 vector genome.

[0158] The AUC profiles of vector preps produced by these methods were remarkably different. Using the producer cell line method, 74% of capsids contained a full genome, represented by the 92S species (**FIG. 6B**). 19% were empty capsids, with the remainder containing a fragmented genome (75S species, 7%). In contrast, 82% of the capsids produced by the triple transfection method were empty, 64S species (**FIG. 6C**), with 11% of capsids containing a fragmented genome (76S and 84S species) and only 8% of capsids having a full genome (94S).

[0159] These results demonstrate that vector preparations generated using the producer cell line technology may have high quality, containing predominantly capsids with a full genome. The vast majority of capsids produced by the triple transfection method are empty, with a greater proportion of capsids having a fragmented genome. These results also highlight the ability of the AUC method to resolve capsids with fragmented genomes, in addition to full genome-containing and empty capsids. Moreover, they illustrate the power of the AUC method in evaluating the quality and homogeneity of vector preparations.

Example 4: Use of AUC to assess removal of empty capsids from vector preps

[0160] The AUC method was evaluated as a tool to monitor removal of empty capsids using chromatographic methods (for methods, see Qu *et al.* (2007) *J. Virol. Methods,* 140:183-92). Separation of empty and genome-containing capsids was performed using anion exchange chromatography (**FIG. 7A**). AUC was performed on the resolved peaks to demonstrate that

the genome-containing rAAV2 particles were enriched in the later fractions eluted from the resin ("Full Genome Capsid" in **FIG. 7A**).

[0161] As shown in **FIG. 7B**, AUC analysis revealed that genome-containing capsids represented 94% of the vector prep upon elution from the column. This later fraction yielded a single peak with a sedimentation coefficient of 92S. In contrast, the rAAV2 vector prep prior to the chromatographic step (**FIG. 7C**) had a substantial level of empty capsids. AUC analysis revealed two peaks with S values of 63 and 93, with the 63S peak (empty capsid) representing 52% of the total capsid population. These results show that chromatographic methods are highly effective in removing empty capsids from AAV vector preparations. Importantly, they demonstrate the utility of applying the AUC method to evaluate vector quality upon purification. The AUC method is a useful tool for evaluating different vector purification protocols or techniques.

Example 5: Assessment of viral genome integrity by the AUC method

[0162] As illustrated in Example 3, AAV vector preparations may contain capsids packaged with fragmented genomes, in addition to full genome and empty species. A major problem in generating homogeneous AAV preparations for therapeutic or research applications is the presence of capsids with fragmented genomes, which may result in aberrant or absent expression of transgenes of interest. Indeed, heterogeneity associated with AAV vector preps has been reported to result from packaging of fragmented genomes or AAV-encapsidated DNA impurities. (Kapranov *et al.* (2012) *Hum. Gene Ther.*, 23:46-55). Therefore, AUC was evaluated as a tool to quantify the aberrant packaging of fragmented genomes in rAAV vector preps.

[0163] Because it is not possible to determine empirically the extinction coefficient of fragmented genomes of unknown size and sequence, a relationship between S value and genome size was established. To achieve this, rAAV vector preps with encapsidated viral genomes of known size were analyzed by AUC, and their corresponding S values were determined, as shown in Table 5. A standard curve was then generated to correlate genome size and S value (**FIG. 8**). This demonstrated a highly linear relationship (R^2=0.9978) between sedimentation coefficient and genome size.

Table 5. S values for rAAV vectors with known genome size.

Predicted Trend Line		Calculated values	
Sedimentation coefficient (S)	**Genome size (# NT)**	**MW**	**Extinction coefficient (260 nm)**
(y)	**(x)**		
Empty capsid	N/A		3.72E+06
74	880	2.7E+05	9.17E+06
78	1421	4.4E+05	1.25E+07
82	1961	6.1E+05	1.59E+07
84	2232	6.9E+05	1.75E+07
88	2772	8.6E+05	2.09E+07
92	3313	1.0E+06	2.42E+07
96	3853	1.2E+06	2.76E+07
100	4394	1.4E+06	3.09E+07
104	4934	1.5E+06	3.43E+07
108	5475	1.7E+06	3.76E+07

[0164] To demonstrate the utility of AUC to detect genome fragments, a self-complementary vector comprising AAV2 ITRS, a minimal CBA promoter, and an EGFP transgene was packaged into an AAV9 capsid (AAV2/9minCBAEGFP; see schematic in **FIG. 9A**). The vector particles were purified to eliminate empty capsids and analyzed by AUC. The standard curve was then used to assign genome size to each of the resolved genome containing capsids. Approximately 25% of the vector prep sedimented as a 101S species, representing an encapsidated genome of ~ 4.3kb (**FIG. 9A**). This 101S peak represented the double stranded dimeric vector genome, which has a predicted size of ~4.3kb. However, the majority of the vector prep (75%), sedimented with an S value of 82, which corresponds to a vector genome size of ~ 2kb (**FIG. 9A**), consistent with packaging of the single stranded monomer. The packaging of monomeric genomes with self-complementary vectors is well documented and is often a result of inadvertent terminal resolution at spurious "trs like" sequences despite the presence of an ITR with a mutated D sequence (McCarty *et al.* (2001) *Gene Ther.,* 8:1248-54).

[0165] **FIG. 9B** shows an alkaline Southern blot of the same vector, scAAV9 EGFP, which revealed two vector populations with genomes ~ 4.3kb and ~2kb in size, corroborating the AUC data in **FIG. 9A**. The Southern blot also confirmed that the monomeric viral genome was preferentially packaged over the dimeric genome. Interestingly, AUC analysis of a single stranded AAV9 EGFP vector (~4kb) revealed a single predominant peak with a measured S value of 99S, corresponding to approximately 4.1kb by the standard curve and 84% of capsid

abundance (**FIG. 9C**). These results suggest that single stranded AAV vectors may be packaged in a more homogeneous manner than double stranded vectors. Again, in agreement with the AUC method, Southern blot analysis of this vector prep revealed homogeneous encapsidation of a viral genome of the predicted size of ~4kb (lane 2, **FIG. 9B**). These results demonstrate that the AUC method may be used to measure the size of AAV vector genomes, yielding genome size data in agreement with the standard Southern blotting technique. Using the AUC method, single stranded AAV vectors were found to produce more homogeneous vector preparations than double stranded ones. These results show that the AUC method is a powerful tool to identify and quantify capsid species with incomplete genomes from vector preparations.

Example 6: Use of AUC to assess factors that influence packaging of vector genomes

[0166] The AUC method was next used as a tool to identify factors that influence the packaging of intact AAV vector genomes.

[0167] As discussed in Example 3, the production of rAAV vectors by transient transfection methods requires the use of three plasmids including a rep/cap helper, an ITR vector plasmid, and a pAd helper (see **FIG. 4**). AUC was used to assess the effect of the rep/cap helper on vector genome packaging for both single stranded and self-complementary AAV vectors. First, a self-complementary AAV vector harboring an EGFP transgene (**FIG. 10A**) was produced using one of two methods. In the first method (**FIG. 10B**), a helper plasmid was used in which rep 78/68 expression was driven by the endogenous p5 promoter ("WT Rep" construct). In the second method (**FIG. 10C**), the helper was modified such that 78/68 expression was reduced by moving the p5 promoter downstream of the cap2 sequence as well as mutating the TATA box ("pHLP Rep" construct). The full scAAV2 EFGP capsid was predicted to have a sedimentation coefficient of 100S in dimeric genome form and 80S in monomeric genome form (**FIG. 10A**).

[0168] AUC analysis of these scAAV2EGFP vector preps revealed a significant difference in vector genome packaging. In the presence of reduced rep78/68 (pHLP), more than half (55%) of the vector prep contained dimeric genomes, represented by the 100S species (**FIG. 10C**). This was the expected sedimentation coefficient for a capsid containing a dimeric

genome of 4.4kb. In contrast, the scAAV2EGFP prep generated with the full complement of rep78/68 had significantly less packaged dimeric genomes (26%), with the majority of the capsids containing monomeric genomes and sedimenting at 80S (**FIG. 10B**). These results uncovered a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid, leading to reduced rep 78/68 protein levels.

[0169] A single stranded AAV5 Factor IX vector, AAV5FIX, (**FIG. 11A-B**) and a single stranded AAV5hSMN vector (**FIG. 11C-D**) were generated using rep/cap helpers that differed in rep expression as described above, but cap sequences of AAV2 were replaced by cap sequences of AAV5. Based on the nucleotide size of the FIX expression cassette (4.3kb), the predicted sedimentation coefficient for the AAV5 FIX vector capsid was approximately 101S. AUC analysis of AAV2/5FIX made in the presence of reduced rep78/68 ("pHLP19 Rep") revealed a homogenous profile with the majority of the vector (90%) sedimenting with an S value of the expected size, ~101S (**FIG. 11A**). In contrast, AAV5 FIX vector generated using a rep/cap5 helper expressing wild-type levels of rep 78/68 proteins ("WT Rep") generated a strikingly different AUC profile (**FIG. 11B**). Instead of a predominant peak at 101S, this profile revealed more capsid heterogeneity, with the majority of the AAV5 FIX (80%) sedimenting at a lower S value of 86S, likely representing packaging of a fragmented genome. Moreover, in this vector sample only 15% of the AAV5 FIX vector capsids sedimented at the correct S value of ~ 104S (**FIG. 11B**).

[0170] AAV5SMN vectors made using these same wild-type and mutated p5 rep/cap helpers also had strikingly different AUC profiles. As seen with the single stranded AAV5FIX vectors, AAV5 SMN vectors generated in the presence of reduced rep78/68 showed less heterogeneity by AUC analysis, with a single capsid species sedimenting at the S value of 101S, consistent with packaging of a genome of the predicted size of ~ 4.4kb (**FIG. 11C**). In contrast, the AUC profile for the same vector genome packaged using "wild-type" levels of rep78/68 protein revealed three distinct AAV vector species, with sedimentation coefficients of 100S (predicted S value for the full vector genome of 4400nt), 92S (representing a fragmented genome of approximately 3300nt) and 80S (representing a fragmented genome of 2000 nucleotides) (**FIG. 11D**). These results confirm a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid using two additional AAV vectors.

[0171] Further analysis of the AAV5SMN and AAV5FIX vector preps was performed by Southern blot analysis of vector DNA. In agreement with the AUC method, Southern blot analysis of AAV5SMN generated with wild-type rep78/68 protein levels revealed packaging of full length (4.4kb) and fragmented (less than 4.4 kb) SMN genomes (**FIG. 12A**, lane 1). In contrast, the AAV5SMN vector generated in the presence of reduced rep78/68 protein contained largely capsids with a full length SMN genome (**FIG. 12A**, lane 2). Interestingly, a comparison of the two AAV5FIX vector preps by Southern analysis revealed the presence of a FIX full length genome even when vector was produced in the presence of wild-type levels of rep 78/68 (**FIG. 12B**, lane 2). However, AUC analysis of this AAV5FIX vector (**FIG. 11B**) showed that 80% of the capsids contained a fragmented genome (~ 3000 nucleotides), which were undetected by the FIX probe.

[0172] Further analysis of the FIX vector preps generated under the two experimental conditions was performed by generating probes to discrete regions of the vector plasmid, including regions of the backbone. A map of this vector is provided in **FIG. 13**. **FIG. 14** shows Southern blotting analyses using these probes to compare these FIX vector preps generated under different conditions. As shown in **FIG. 14A**, both vector preparations (pHLP rep, lane 1; WT rep, lane 2) contained the hFIX transgene. However, **FIG. 14B** lane 2confirms that the vector genome species sedimenting at 86S observed in the WT Rep preparation (**FIG. 11B**) was a ~ 3kb fragment. Moreover, this species reacted with an Amp^R specific probe (**FIG. 14B**, lane 2), suggesting that packaging upstream of the 5'ITR had occurred in a rep dependent manner. In contrast, there was no evidence of an Amp^R containing fragment in the rAAV5 FIX vector preps that were generated in the presence of reduced levels of rep 68 / 78 (**FIG. 14B**, lane 1).

[0173] DNA impurities in the AAV FIX preps were also assessed by Q-PCR using primers and probes specific for Amp^R. By Q-PCR, approximately 35% Amp^R titer was detected in AAV5FIX vector preps generated in the presence of "wt" rep, in contrast to less than 1% when the same vector plasmid was used to generate AAV5FIX vector in the presence of reduced rep68/78 (data not shown). These results underscore the utility of AUC analysis for revealing the presence of packaged genomes that would otherwise go undetected by gene specific Southern blot analysis.

[0174] The packaging capacity of AAV vectors has been studied extensively, and although numerous reports have demonstrated successful transduction with vectors packaging oversized AAV genomes, the latter have been shown to be fragmented into subgenomic – length DNA. To further explore the applicability of the AUC method, the heterogeneity of AAV vectors produced using oversized genomes was evaluated. An expression cassette harboring the full length CBA promoter driving expression of the β-phosphodiesterase transgene was packaged as an oversized genome of 5.4kb (**FIG. 15A**) or as a wild type size genome of 4.6kb (**FIG. 15B**). To generate the 4.6kb genome, the CBA promoter was truncated by reducing the size of the intron as previously reported (Gray, SJ *et al.*, (2011) *Hum. Gene Ther.* 22(9):1143-1153).

[0175] As shown in **FIG. 15A**, the AUC profile of the AAV vector prep generated using the oversized vector genome demonstrated that nearly half of the vector prep sedimented as a 93S species, consistent with packaging a fragmented vector genome of approximately 3.5kb. 30% of the preparation was represented by another sub-genomic vector species of approximately 4.9kb sedimenting at 105S. There was no evidence of packaging of a full-length 5.4kb genome, which was predicted to sediment at 108-109S. In contrast, AUC analysis revealed that the same transgene under control of the abbreviated CBA promoter sedimented predominantly as a vector species of 102S, consistent with packaging of the predicted, full-length vector genome of 4.6 kb (**FIG. 15B**). These results demonstrate the utility of AUC analysis in profiling AAV vectors with oversized genomes, and this profiling is critical, given the observed incidence of genome fragmentation.

[0176] This example demonstrated that the AUC method is highly effective in analyzing the genome size of AAV vector capsids in a heterogeneous preparation. By resolving genome-containing capsids by size (*e.g.,* dimeric and monomeric genomes, or partial fragments thereof), the AUC method represents a powerful tool for assaying the quality of AAV vector preps produced under different conditions. Moreover, the results from three distinct vector systems demonstrated that the AUC method is widely useful for quality control and optimization of conditions to yield improved AAV vector preparations. Importantly, the AUC method is able to detect fragmented genomes that are not detectable by Southern blot analysis. Whereas Southern blotting relies on the presence of DNA probe sequence for detection, the AUC method is sequence-independent. The AUC method has also been demonstrated to be an

effective tool in analyzing oversized AAV genomes. In total, these results demonstrate the highly advantageous and effective implementation of AUC methods to analyze multiple types of AAV vector preparations, which have been found to display dramatically variable effects on genome packaging.

CLAIMS

What is claimed is:

1. A method of characterizing a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

2. A method to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and

c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes.

3. The method of claim 2 further comprising integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

4. A method to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

5. A method of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, and

d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

6. A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

7. A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

8. A method of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

9. A method of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method of any one of claims 5-8, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

10. The method of any one of claims 4-9, wherein the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles.

11. The method of any one of claims 4-9, wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes.

12. The method of claim 11, wherein the capsid particles comprising variant sized genomes comprises truncated genomes, aggregates, recombinants and/or DNA impurities.

13. A method of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation.

14. The method of claim 13, wherein the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes.

15. The method of claim 14, wherein the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities.

16. The method of any one of claims 13-15 further comprising integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles.

17. A method of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the method of claim 16, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

18. The method of any one of claims 1-17, wherein the sedimentation of recombinant viral particles is monitored by absorbance.

19. The method of claim 18, wherein the absorbance is at about 230 nm, 260 nm or 280 nm.

20. The method of claim 18 or 19, wherein the absorbance is at about 260 nm.

21. The method of any one of claims 1-20, wherein the sedimentation of recombinant viral particles is monitored by interference.

22. The method of claim 21, wherein the interference is Rayleigh interference.

23. The method of any one of claims 1-22, wherein the preparation is an aqueous solution.

24. The method of claim 23, wherein the aqueous solution comprises a pharmaceutical formulation.

25. The method of claim 23 or 24, wherein the aqueous solution comprises a buffer.

26. The method of claim 25, wherein the buffer is at physiological pH.

27. The method of claim 25 or 26, wherein the buffer is at physiological osmolality.

28. The method of any one of claims 24-27, wherein the pharmaceutical formulation comprises phosphate buffered saline (PBS).

29. The method of claim 28, wherein the PBS has pH of about 7.2 and an osmolality of about 300 mOsm/L.

30. The method of any one of claims 24-30, wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

31. The method of any one of claims 1-30, wherein the C(S) values are determined by an algorithm that comprises Lamm equation solutions.

32. The method of claim 31, wherein the algorithm is the SEDFIT algorithm.

33. The method of any one of claims 1-32, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge.

34. The method of any one of claims 1-32, wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell.

35. The method of any one of claims 1-33, wherein sedimentation is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell.

36. The method of claim 34, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window.

37. The method of any one of claims 33-36, wherein at least 30 scans are used to monitor sedimentation of recombinant viral particles.

38. The method of claim 37, wherein about 30 scans are used to monitor sedimentation of recombinant viral particles.

39. The method of claim 37, wherein about 30 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

40. The method of claim 37, wherein about 30 to about 50 scans are used to monitor sedimentation of recombinant viral particles.

41. The method of claim 37, wherein about 50 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

42. The method of claim any one of claims 31-41, wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68.

43. The method of claim 42, wherein the regularization is a second derivative regularization.

44. The method of claim 42, wherein the regularization is Max entropy regularization.

45. The method of any one of claims 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.90.

46. The method of any one of claims 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.99.

47. The method of any one of claim 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68.

48. The method of any one of claims 31-47, wherein the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software.

49. The method of claim 48, wherein resolution is about 200S to about 1000S.

50. The method of claim 48 or 49, wherein resolution is about 200S.

51. The method of any one of claims 48-50, wherein S min is about 1.

52. The method of any one of claims 48-51, wherein Smax is about 100S to about 1000S.

53. The method of any one of claims 48-51, wherein Smax is about 200S to about 5000S.

54. The method of any one of claims 48-51, wherein Smax is about 200S.

55. The method of any one of claims 48-54, wherein the frictional ratio is left to float to a value determined by centrifugation software.

56. The method of claim any one of 48-54, wherein the frictional ratio is about 1.0.

57. The method of any one of claims 31-56, wherein radial invariant (RI) and time invariant (TI) noise subtractions are applied.

58. The method of any one of claims 1-57, wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds.

59. The method of claim 58, wherein the sedimentation of recombinant viral particles is monitored about every 10 seconds.

60. The method of claim 58, wherein the sedimentation of recombinant viral particles is monitored about every 60 seconds

61. The method of any one of claims 1-60, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm.

62. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 10,000 rpm.

63. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 10,000 rpm to about 20,000 rpm.

64. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 15,000 rpm to about 20,000 rpm.

65. The method of any one of claims 1-64, wherein the boundary sedimentation velocity is performed at about 4°C to about 20 °C.

66. The method of claim 65, wherein the boundary sedimentation velocity is performed at about 4°C.

67. The method of any one of claims 1-66, wherein the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle.

68. A method of evaluating a process for the production of recombinant viral particles comprising the method of any one of claims 1 to 67, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles.

69. The method of claim 68, wherein the viral particles are rAAV particles.

70. The method of claim 69, wherein the rAAV particles are produced from a producer cell line.

71. The method of claim 69, wherein the rAAV particles are produced by triple transfection of i) nucleic acid encoding AAV rep and cap, ii) rAAV vector sequences, and iii) nucleic acid encoding adenovirus helper functions.

72. The method of claim 69, wherein the recombinant viral particles are produced by an AAV/HSV hybrid

73. The method of claim 69, wherein the recombinant viral particles are produced from a baculovirus cell.

74. The method of claim 69, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell.

75. The method of claim 69, wherein the recombinant viral particles are produced by introduction of one or more nucleic acids encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell, wherein the one or more nucleic acids are introduced to the cell using a recombinant helper virus.

76. The method of claim 75, wherein the recombinant helper virus is an adenovirus or a herpes simplex virus.

77. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding adenovirus vector sequences and adenovirus replication and packaging sequences to a suitable host cell.

78. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding lentivirus vector sequences and/or lentivirus replication and packaging sequences to a suitable host cell.

79. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding HSV vector sequences and/or HSV replication and packaging sequences to a suitable host cell.

80. A method for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising

a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant viral particles;

c) isolating the recombinant viral particles produced by the host cell; and

d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the methods of any one of claims 1 to 79.

81. The method of claim 80, wherein the p5 promoter is located 3' to the rep and/or cap coding region.

82. The method of claim 80 or 81, wherein the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

83. The method of any one of the preceding claims wherein the recombinant viral particles have been purified using one or more purification steps.

84. The method of claim 67, 69-76, 80-83 wherein the recombinant viral particles comprise a self-complementary AAV (scAAV) genome.

85. The method of claim 84, wherein the method is used to detect the presence of recombinant viral particles comprising the monomeric form of a scAAV genome or the dimeric form of a scAAV genome.

86. The method of any one of claims 67, 69-76, 80-85,wherein the recombinant viral particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an

AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1).

87. The method of any one of claims 67, 69-76, 80-86, wherein the recombinant viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR.

88. The method of claim 86 or 87, wherein the AAV capsid comprises a tyrosine mutation or a heparin binding mutation,

89. The method of claim 67 or 68, where the recombinant viral particle is a recombinant adenoviral particle.

90. The method of claim 89, wherein the recombinant adenoviral particle comprises an capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, or porcine Ad type 3.

91. The method of claim 90, wherein the recombinant adenoviral particle comprises a variant of an adenovirus serotype 2 capsid or a variant of an adenoviral serotype 5 capsid.

92. The method of claim 67 or 68, where the recombinant viral particle is a recombinant lentiviral particle.

93. The method of claim 92, wherein the recombinant lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114 or variants therein.

94. The method of claim 67 or 68, where the recombinant viral particle is a rHSV particle.

95. The method of claim 94, wherein the HSV particle is an HSV-1 particle or an HSV-2 particle.

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DOCUMENT MADE AVAILABLE UNDER THE PATENT COOPERATION TREATY (PCT)

International application number:		**PCT/US2016/013947**
International filing date:		**19 January 2016 (19.01.2016)**
Document type:		**Certified copy of priority document**
Document details:	Country/Office:	**US**
	Number:	**62/105,714**
	Filing date:	**20 January 2015 (20.01.2015)**
Date of receipt at the International Bureau:		**01 February 2016 (01.02.2016)**

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-*bis*)

34, chemin des Colombettes
1211 Geneva 20, Switzerland
www.wipo.int

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT/US16/13947
International Application No.

19 JAN 2016(19.01.2016)
International Filing Date

RO/US
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 159792010340

Box No. I TITLE OF INVENTION

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GENZYME CORPORATION
500 Kendall Street
Cambridge, Massachusetts 02142
United States of America

Telephone No.

Facsimile No.

Applicant's registration No. with the Office

E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so.

☐ as advance copies followed by paper notifications; or ☐ exclusively in electronic form (no paper notifications will be sent).

E-mail address:

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☒ all designated States ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

DONAHUE, Brian A.
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Agent's registration No. with the Office 58,206

E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so.

☒ as advance copies followed by paper notifications; or ☐ exclusively in electronic form (no paper notifications will be sent).

E-mail address: patentdocket@mofo.com

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

O'RIORDAN, Catherine R.
c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807
United States of America

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☒ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

BURNHAM, Brenda
c/o Sanofi
55 Corporate Drive
Mail Code: 55A-505A
Bridgewater, New Jersey 08807
United States of America

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☒ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. *If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided,* ***the space is insufficient*** *to furnish all the information: in such case, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

(i) ***if more than one person is to be indicated as applicant and/or inventor*** *and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

(ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication* ***"the States indicated in the Supplemental Box"*** *is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

(iii) *if, in Box No. II or in any of the sub-boxes of Box No. III,* ***the inventor or the inventor/applicant is not inventor for the purposes of all designated States****: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

(iv) *if, in addition to the agent(s) indicated in Box No. IV, there are* ***further agents****: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

(v) *if, in Box No. VI, there are* ***more than three earlier applications whose priority is claimed****: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*

2. *If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such case, write the name or two-letter code of each designated State concerned and the indication "****patent of addition,****" "****certificate of addition,****" "****inventor's certificate of addition****" or "****utility certificate of addition,****" the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other parent grant or the date of filing of the parent application (Rules 4.11(a)(i) and 49bis.1(a) or (b)).*

3. *If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such case, write "United States of America" or "US" and the indication "****continuation****" or "****continuation-in-part****" and the number and the filing date of the parent application (Rules 4.11(a)(ii) and 49bis.1(d)).*

Continuation of Box No. IV:

Lisa A. Amii, 48,199; Mehran Arjomand, 48,231; Max Banko, 72,557; Erol C. Basol, 65,150; Vincent J. Belusko, 30,820; Shouvik Biswas, 68,439; David A. Blumenthal, 26,257; Jonathan Bockman, 45,640; Kimberly A. Bolin, 44,546; Yuchen Cao, 73,383; Robert K. Cerpa, 39,933; John Chapman, 70,339; Alex Chartove, 31,942; Peng Chen, 43,543; Anita Choi, 71,792; Thomas E. Ciotti, 21,013; Joshua Crawford, 72,137; Brian A. Donahue, 58,206; Christopher B. Eide, 48,375; Cory Ellison, 72,195; David L. Fehrman, 28,600; Kathleen Fitzpatrick, 72,140; James Frei, 71,458; Takamitsu Fujiu, 63,971; Hector G. Gallegos, 40,614; Mayra L. Garcia, 71,597; Christopher Gloria, 71,907; Brian B. Ho, 60,199; Matthew Hollister, 69,313; Candice Hsu, 66,237; Liang Huang, 67,016; Jessica Jarecki, 73,669; Kevin Jones, 73,475; Stephen C. Jones, 68,808; Parisa Jorjani, 46,813; John Karlen, 72,763; David S. Kim, 57,143; Matthew I. Kreeger, 56,398; Glenn M. Kubota, 44,197; Parker Kuhl, 69,867; Jane Kuzelka, 70,963; Christine Lekutis, 51,934; Catherine O. Liang, 61,778; Joanna Liebes, 71,499; Otis Littlefield, 48,751; Victor Liu, 70,880; Jordan Lofdahl, 59,163; Skyler Lund, 72,976; Cong Luo, 68,120; Mika Mayer, 47,777; Katharine E. McElhone, 57,462; Meghan McLean, 73,248; Bradley J. Meier, 44,236; Elizabeth Cary Miller, 54,708; Amy Motomura, 70,897; James J. Mullen, III, 44,957; Kate H. Murashige, 29,959; Kenneth W. Newton, 73,157; Jean Nguyen, 71,051; Martin Noonen, 44,264; Randy Omid, 67,439; Christina Onufryk, 59,842; Desmond O'Sullivan, 67,576; Aaron Parker, 74,355; Jillian Pesin-Fulop, 63,950; Catherine M. Polizzi, 40,130; Karen Potter, 57,254; Yan Qi, 73,252; Shannon Reaney, 52,285; Roberto K. Rodriguez, 66,244; Madhuri Roy, 64,497; Brian Schar, 45,076; Lisa N. Silverman, 69,080; Michael A. Stallman, 29,444; Jonathan Statman, 71,638; Michael Swett, 68,132; Zong-Qiang Bill Tian, 61,753; Connie C. Tong, 52,292; Patricia Tsao, 50,713; Diek Van Nort, 60,777; Filip Vanevski, 71,802; Nathan Vogler, 71,568; Michael R. Ward, 38,651; Ethan Weiner, 73,258; Amanda T. White, 73,395; Jennifer White, 72,269; Robert Wu, 60,024; Walter Wu, 50,816; Jian Xiao, 55,748; David T. Yang, 44,415; Hain-Ann Hsueh Yang, 65,993; Alex Yap, 60,609; Boyoung Yeum, 73,803; Peter J. Yim, 44,417; Bu Yin, 68,930; and Jie Zhou, 52,395

Novartis Exhibit 1002, Page 257 of 591

Box No. V DESIGNATIONS

The filing of this request **constitutes under Rule 4.9(a) the designation** of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

However,

☐ DE Germany **is not designated** for any kind of national protection

☐ JP Japan **is not designated** for any kind of national protection

☐ KR Republic of Korea **is not designated** for any kind of national protection

(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)

Box No. VI PRIORITY CLAIM AND DOCUMENT

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 20 January 2015 (20.01.2015)	62/105,714	US		
item (2)				
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

Furnishing the priority document(s):

☒ The **receiving Office** is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application(s) was filed with the receiving Office which, for the purposes of this international application, is the receiving Office)* identified above as:

☒ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

☐ The **International Bureau** is requested to obtain from a digital library a certified copy of the earlier application(s) identified above, using, where applicable, the access codes(s) indicated below *(if the earlier application(s) is available to it from a digital library)*:

☐ item (1) access code _____ ☐ item (2) access code _____ ☐ item (3) access code _____ ☐ other, see Supplemental Box

Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (_____). *(See also the Notes to Box No. VI; further information **must** be provided to support a request to restore the right of priority.)*

Incorporation by reference: where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if more than one International Searching Authority is competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / EP

Continuation of Box No. VII USE OF RESULTS OF EARLIER SEARCH, REFERENCE TO THAT SEARCH

☐ The ISA indicated in Box No. VII is requested to take into account the results of the earlier search(es) indicated below (*see also Notes to Box VII; use of results of more than one earlier search*).

Filing date *(day/month/year)* | Application Number | Country *(or regional Office)*

☐ **Statement (Rule 4.12(ii))**: this international application is the same, or substantially the same, as the application in respect of which the earlier search was carried out except, where applicable, that it is filed in a different language.

☐ **Availability of documents**: the following documents are available to the ISA in a form and manner acceptable to it and therefore do not need to be submitted by the applicant to the ISA (Rule 12*bis*.1(f)):

- ☐ a copy of the results of the earlier search,*
- ☐ a copy of the earlier application,
- ☐ a translation of the earlier application into a language which is accepted by the ISA,
- ☐ a translation of the results of the earlier search into a language which is accepted by the ISA,
- ☐ a copy of any document cited in the results of the earlier search. *(If known, please indicate below the document(s) available to the ISA):*

☐ **Transmit copy of results of earlier search and other documents** *(where the earlier search **was not** carried out by the ISA indicated above but by the same Office as that which is acting as the receiving Office)*: the **receiving Office** is requested to prepare and transmit to the ISA (Rule 12*bis*.1(c)):

- ☐ a copy of the results of the earlier search,*
- ☐ a copy of the earlier application,
- ☐ a copy of any document cited in the results of the earlier search.

* Where the results of the earlier search are neither available from a digital library nor transmitted by the receiving Office, the applicant is required to submit them to the receiving Office (Rule 12*bis*.1(a)) (*See item 11. in the check-list and also Notes to Box No. VII*).

Filing date *(day/month/year)* | Application Number | Country *(or regional Office)*

☐ **Statement (Rule 4.12(ii))**: this international application is the same, or substantially the same, as the application in respect of which the earlier search was carried out except, where applicable, that it is filed in a different language.

☐ **Availability of documents**: the following documents are available to the ISA in a form and manner acceptable to it and therefore do not need to be submitted by the applicant to the ISA (Rule 12*bis*.1(f)):

- ☐ a copy of the results of the earlier search,*
- ☐ a copy of the earlier application,
- ☐ a translation of the earlier application into a language which is accepted by the ISA,
- ☐ a translation of the results of the earlier search into a language which is accepted by the ISA,
- ☐ a copy of any document cited in the results of the earlier search. *(If known, please indicate below the document(s) available to the ISA):*

☐ **Transmit copy of results of earlier search and other documents** *(where the earlier search **was not** carried out by the ISA indicated above but by the same Office as that which is acting as the receiving Office)*: the **receiving Office** is requested to prepare and transmit to the ISA (Rule 12*bis*.1(c)):

- ☐ a copy of the results of the earlier search,*
- ☐ a copy of the earlier application,
- ☐ a copy of any document cited in the results of the earlier search.

* Where the results of the earlier search are neither available from a digital library nor transmitted by the receiving Office, the applicant is required to submit them to the receiving Office (Rule 12*bis*.1(a)) (*See item 11. in the check-list and also Notes to Box No. VII*).

☐ Further earlier searches are indicated on a continuation sheet.

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*:			Number of declarations
☐	Box No. VIII (i)	Declaration as to the identity of the inventor :	
☐	Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent :	
☐	Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application :	
X	Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America) :	2
☐	Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty :	

Box No. IX CHECK LIST for EFS-Web filings - this sheet is only to be used when filing an international application with RO/US via **EFS-Web**

This international application **contains** the following:	Number of sheets
(a) request form PCT/RO/101 (including any declarations and supplemental sheets)	7
(b) description (excluding any sequence listing part of the description, see (f), below)	74
(c) claims	13
(d) abstract	1
(e) drawings (if any)	18
(f) sequence listing part of the description in the form of an **image file** (e.g. PDF)	
Total number of sheets (including the sequence listing part of the description if **filed as an image file**)	113

(g) sequence listing part of the description

- [ ] filed in the form of an **Annex C/ST.25 text file**
- [ ] WILL BE filed separately on physical data carrier(s), on the same day and in the form of an **Annex C/ST.25 text file**

Indicate type and number of physical data carrier(s)

This international application is **accompanied by** the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item):*	Number of items
1. [x] fee calculation sheet	1
2. [ ] original separate power of attorney	
3. [ ] original general power of attorney	
4. [ ] copy of general power of attorney; reference number:	
5. [ ] priority document(s) identified in Box No. VI as item(s)	
6. [ ] Translation of international application into *(language)*:	
7. [ ] separate indications concerning deposited microorganism or other biological material	
8. [ ] *(only where item (f) is marked in the left column)* copy of the sequence listing in electronic form (Annex C/ST.25 text file) not forming part of the international application but **furnished only for the purposes of international search** under Rule 13*ter*	
9. [ ] *(only where item (f) (in the left column) and item 8 (above) are marked)* a statement confirming that "the information recorded in electronic form submitted under Rule 13*ter* is identical to the sequence listing as contained in the international application" as filed via EFS-Web:	
10. [ ] copy of results of earlier search(es) (Rule 12*bis*.1(a))	
11. [x] other *(specify)*: Transmittal Letter and Certificate of Electronic Filing.	2

Figure of the drawings which should accompany the abstract: 1B

Language of filing of the international application: English

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

/Brian A. Donahue/
Brian A. Donahue, Agent for Applicant(s)
MORRISON & FOERSTER LLP

Attorney Reg. No. (when applicable): 58,206

19-01-2016

For receiving Office use only

1. Date of actual receipt of the purported international application: 19 JAN 2016		2. Drawings: [ ] received: [ ] not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		
4. Date of timely receipt of the required corrections under PCT Article 11(2):		
5. International Searching Authority (if two or more are competent): ISA / EP	6. [ ] Transmittal of search copy delayed until search fee is paid	

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/105,714, filed on January 20, 2015, the content of which is hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to methods to characterize recombinant viral vectors; *e.g.*, recombinant adeno-associated viral (AAV) particles, recombinant adenoviral (rAd) particles, recombinant lentiviral particles and recombinant Herpes simplex viral (rHSV) particles using analytical ultracentrifugation.

BACKGROUND OF THE INVENTION

[0003] Recombinant viruses show great promise and utility as a vehicle to deliver therapeutic nucleic acids for gene therapy applications. A number of different recombinant viruses are used in these gene therapy applications based on a number of factors including the size of the nucleic acid to be delivered, the target cell or tissue to deliver the nucleic acid, the need for short or long term expression of the therapeutic nucleic acid, and integration of the therapeutic nucleic acid into the recipient's genome. Examples of viruses used in gene therapy applications include adeno-associated virus (AAV), adenovirus, lentivirus and herpes simplex virus (HSV).

[0004] The generation of recombinant viral vectors for the clinic requires an analytical method that monitors drug product quality with regard to homogeneity, purity and consistency of manufacturing, yet to date no method to support such a characterization has been established. Typically, the DNA content of recombinant viral DNA viral vectors is measured by Southern blot analysis using a sequence specific probe. Viral capsids or envelopes may be characterized by immunoassay using an antibody that binds specifically to a capsid or envelope protein of a particular recombinant virus. For example, Steinbach, S *et al.*, (1997) *J. Gen. Virol.*, 78:1453-

1462 provides an immunoassay for rAAV serotypes. What is needed is a generic assay to characterize recombinant viral preparations regardless of the nucleic acid sequence of the recombinant viral genome or the serotype of the capsid.

[0005] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0006] In some aspects, the invention provides methods of characterizing a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

[0007] In some aspects, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

[0008] In some aspects, the invention provides methods to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of

recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

[0009] In some aspects, the invention provides, methods of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, and d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

[0010] In some aspects, the invention provides methods of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

[0011] In some aspects, the invention provides methods of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

[0012] In some aspects, the invention provides methods of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

[0013] In some aspects, the invention provides, methods of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method of any one of claims 5-8, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the

preparation of recombinant viral particles. In some embodiments, the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles. In some embodiments, the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes. In some embodiments, the capsid particles comprising variant sized genomes comprise truncated genomes, aggregates, recombinants and/or DNA impurities.

[0014] In some aspects, the invention provides methods of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation. In some embodiments, the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes. In some embodiments, the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles.

[0015] In some aspects, the invention provides methods of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the above method, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

[0016] In some embodiments of the above aspects, sedimentation of recombinant viral particles is monitored by absorbance. In some embodiments, the absorbance is at about 230 nm, 260 nm or 280 nm. In some embodiments, the absorbance is at about 260 nm. In some

embodiments, sedimentation of recombinant viral particles is monitored by interference. In some embodiments, the interference is Rayleigh interference.

[0017] In some embodiments of the above aspects, the preparation is an aqueous solution. In further embodiments, the aqueous solution comprises a pharmaceutical formulation. In some embodiments, the aqueous solution comprises a buffer. In some embodiments, the buffer is at physiological pH. In some embodiments, the buffer is at physiological osmolality. In some embodiments, the pharmaceutical formulation comprises phosphate buffered saline (PBS). In some embodiments, the PBS has pH of about 7.2 and an osmolality of about 300 mOsm/L. In some embodiments, the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

[0018] In some embodiments of the above aspects, the C(S) values are determined by an algorithm that comprises Lamm equation solutions. In some embodiments, the algorithm is the SEDFIT algorithm. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge; for example, the sector may be a portion of the ultracentrifuge comprising a detection system. In some embodiments, the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until recombinant viral particles sediment to the bottom of ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window.

[0019] In some embodiments, the radial concentration is recorded for at least about any of 0.5 hours, 0.75 hours, 1.0 hours, 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, or 5.0 hours. In some embodiments, the radial concentration is recorded for about 1.0 hour. In some embodiments, the radial concentration is recorded for about 1.2 hours. In some embodiments, the radial concentration is recorded from about 0.5 hours to about 2.0 hours. In some embodiments, the radial concentration is recorded from about 1.0 hours to about 2.0 hours.

[0020] In some embodiments of the above aspects, at least 30 scans are used to monitor sedimentation of recombinant viral particles. In some aspects, about 30 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 30 to about 75 scans

are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 30 to about 50 scans are used to monitor sedimentation of recombinant viral particles. In other embodiments, about 50 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

[0021] In some embodiments of the above aspects, a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68. In some embodiments, the regularization is a second derivative regularization. In some embodiments, the regularization is Max entropy regularization. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.90. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.99. In some embodiments, the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68.

[0022] In some embodiments of the above aspects, the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software. In some embodiments, resolution is about 200S to about 1000S. In some embodiments, resolution is about 200S. In some embodiments, S min is about 1. In some embodiments, Smax is about 100S to about 1000S. In other embodiments, Smax is about 200S to about 5000S. In other embodiments, Smax is about 200S. In some embodiments, the frictional ratio is left to float to a value determined by centrifugation software. In some embodiments, the frictional ratio is about 1.0. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

[0023] In some embodiments of the above aspects, the sedimentation of recombinant viral particles is monitored about every 10-60 seconds. In some embodiments, sedimentation of recombinant viral particles is monitored (*e.g.*, scanned) about every 10 seconds. In other embodiments, the sedimentation of recombinant viral particles is monitored about every 60 seconds. In some embodiments, the sedimentation velocity of recombinant viral during ultracentrifugation is determined by monitoring the sedimentation of recombinant viral particles once in more than about every 15 seconds, 30 seconds, 45 seconds, 1 minute (60 seconds), 2

minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes.

[0024] In some embodiments of the above aspects, the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm. In some embodiments, the boundary sedimentation velocity is performed at about 3,000 rpm to about 10,000 rpm. In other embodiments, the boundary sedimentation velocity is performed at about 10,000 rpm to about 20,000 rpm. In other embodiments, the boundary sedimentation velocity is performed at about 15,000 rpm to about 20,000 rpm.

[0025] In some embodiments of the above aspects, the boundary sedimentation velocity is performed at about 4°C to about 20 °C. In some embodiments, the boundary sedimentation velocity is performed at about 4°C.

[0026] In some embodiments of the above aspects, the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle. In some embodiments, the recombinant viral particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the recombinant viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In other embodiments, the recombinant viral particle is a recombinant adenoviral particle. In some embodiments, the recombinant adenoviral particle comprises an capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine

Ad, or porcine Ad type 3. In some embodiments, the recombinant adenoviral particle comprises a variant of an adenovirus serotype 2 capsid or a variant of an adenoviral serotype 5 capsid. In other embodiments, the recombinant viral particle is a recombinant lentiviral particle. In some embodiments, the recombinant lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114 or variants therein. In other embodiments, the recombinant viral particle is a rHSV particle. In some embodiments, the HSV particle is an HSV-1 particle or an HSV-2 particle.

[0027] In some aspects, the invention provides methods for evaluating a process for the production of recombinant viral particles comprising the method of any one of claims 1 to 67, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles. In some embodiments, the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle. In some embodiments, the rAAV particles are produced from a producer cell line. In other embodiments, the rAAV particles are produced by triple transfection of i) nucleic acid encoding AAV rep and cap, ii) rAAV vector sequences, and iii) nucleic acid encoding adenovirus helper functions. In other embodiments, the recombinant viral particles are produced by an AAV/HSV hybrid. In other embodiments, the recombinant viral particles are produced from a baculovirus cell. In some embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell. In some embodiments, the recombinant viral particles are produced by introduction of one or more nucleic acids encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell, wherein the one or more nucleic acids are introduced to the cell using a recombinant helper virus. In some embodiments, the recombinant helper virus is an adenovirus or a herpes simplex virus. In some embodiments, the recombinant viral particles comprise a self-complementary AAV (scAAV) genome. In some embodiments, the method is

used to detect the presence of recombinant viral particles comprising the monomeric form of a scAAV genome or the dimeric form of a scAAV genome.

[0028] In some embodiments of the above aspect, the recombinant viral particles are produced by transient transfection of nucleic acid encoding adenovirus vector sequences and adenovirus replication and packaging sequences to a suitable host cell. In other embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding lentivirus vector sequences and/or lentivirus replication and packaging sequences to a suitable host cell. In other embodiments, the recombinant viral particles are produced by transient transfection of nucleic acid encoding HSV vector sequences and/or HSV replication and packaging sequences to a suitable host cell.

[0029] In some aspects the invention provides methods for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release recombinant viral particles; c) isolating the recombinant viral particles produced by the host cell; and d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the above methods. In some aspects the invention provides methods for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release recombinant viral particles; c) isolating the recombinant viral particles produced by the host cell; and d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the above methods. In some embodiments, the p5 promoter is located 3' to the rep and/or cap coding region.

In some embodiments, the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

[0030] In some embodiments, of any of the preceding embodiments, the recombinant viral particles have been purified using one or more purification steps.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] **FIGS. 1A and 1B** show that analytical ultracentrifugation (AUC) can be used to characterize recombinant viral vector particles. (**FIG. 1A**) A representative scanning profile of boundary sedimentation velocity depicting the absorbance (260 nm) versus the radius (cm) of an AAV2 mixture over a time interval (T) of 1.2 hours. The AAV2 mixture contained empty capsids ("Empty Cap") and full genome capsids ("Intact Vector"). (**FIG. 1B**) A plot of concentration in units of detection, C(S), versus sedimentation coefficient (Svedberg units, S) showing that AUC can be used to measure the concentration of empty capsids and full genome capsids from an 80%/20% mixture. Each peak is labeled with the particle species and its corresponding sedimentation coefficient (S) and relative abundance (%).

[0032] **FIGS. 2A and 2B** show the AUC profiles of pure populations of empty AAV2 capsids (**FIG. 2A**) and genome-containing AAV2-transgene 1 capsids (**FIG. 2B**). Each peak is labeled with the capsid species and its sedimentation coefficient (S).

[0033] **FIGS. 3A and 3B** show a comparison between interference and absorbance detection methods by AUC. (**FIG. 3A**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a 1:1 mixture of empty and genome-containing capsids generated using interference optical detection. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**FIG. 3B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a 1:1 mixture of empty and genome-containing capsids generated using absorbance optical detection (260 nm). The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0034] **FIG. 4** illustrates the triple transfection method for AAV vector production. The three vectors, containing the gene of interest ("pVector"), AAV Rep and Cap genes ("pHLP"), and adenoviral components ("pIAdeno") are labeled. Note that both genome-containing (labeled with the "ITR-Transgene-ITR" graphic) and empty capsids (blank) are produced.

[0035] **FIG. 5** illustrates the producer cell line method for AAV vector production. As labeled, the HeLa S3 cell line contains integrated Rep, Cap, and Puromycin resistance genes, along with an ITR-flanked transgene of interest. This cell line is infected with adenovirus ("*Ad5*") to stimulate recombinant viral production. Note that both genome-containing (labeled "recombinant viral Vector") and empty capsids are produced, in addition to adenovirus particles.

[0036] **FIGS. 6A, 6B and 6C** shows that vector production by the producer cell line and triple transfection methods yields different vector preparations, as revealed by AUC analysis. (**FIG. 6A**) A schematic of the AAV2-transgene 2 vector and its 3.4 kb genome. (**FIG. 6B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation produced by the producer cell line method. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**FIG. 6C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation produced by the triple transfection method. The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0037] **FIGS. 7A, 7B and 7C** show that the AUC method may be used to monitor the quality and efficacy of vector purification. (**FIG. 7A**) A plot showing the purification of full-genome AAV2-transgene 1 capsids from empty capsids using anion exchange chromatography. Peak fractions corresponding to each species are labeled. (**FIG. 7B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation after elution from the anion exchange column. The sedimentation coefficient and relative abundance (%) for each species are labeled. (**FIG. 7C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a vector preparation before chromatography. The sedimentation coefficient and relative abundance (%) for each species are labeled.

[0038] **FIG. 8** shows the linear relationship between sedimentation coefficient and vector genome size. A standard curve plotting sedimentation coefficient (S) versus genome size is depicted, along with a line of best fit, its formula, and its associated R^2 value.

[0039] **FIGS. 9A, 9B and 9C** show that assessment of capsid genome size using AUC data correlates with assessment of genome size by Southern blot. (**FIG. 9A**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV9 EGFP vector preparation. Single stranded monomeric (82S) and double stranded dimeric (101S) species are labeled with the corresponding sedimentation coefficients and relative abundance values (%). A schematic of the vector is also provided. (**FIG. 9B**) Alkaline Southern blot analysis of the DNA from scAAV9 EGFP (lane 1) and single stranded AAV9 EGFP (lane 2) vector capsids. Corresponding bands are labeled as described in the blot legend. 4.2 and 2.4 kb size standards are provided as labeled. (**FIG. 9C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a single stranded AAV9 EGFP vector preparation. 82S and 99S (full genome) peaks are labeled with the corresponding sedimentation coefficient and relative abundance values (%).

[0040] **FIGS. 10A, 10B and 10C** show that the Rep/Cap promoter position affects genome packaging in recombinant viral vectors produced by the triple transfection method. (**FIG. 10A**) A schematic of the self-complementary scAAV2 EGFP vector, with estimated sedimentation coefficients for the dimeric and monomeric genome species. (**FIG. 10B**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV2 EGFP vector preparation produced using a "wild-type" helper plasmid with the endogenous p5 promoter driving Rep 78/68 expression ("WT Rep"). Peaks for single stranded monomeric (80S) and double stranded dimeric (100S) species are labeled with the corresponding relative abundance values (%). (**FIG. 10C**) A plot of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yields the distribution of sedimentation coefficients for a scAAV2 EGFP vector preparation produced using a "wild-type" helper plasmid with the p5 promoter driving Rep 78/68 expression moved downstream of the cap2 sequence ("pHLP Rep"). Peaks for single

stranded monomeric (82S) and double stranded dimeric (100S) species are labeled with the corresponding relative abundance values (%).

[0041] **FIGS. 11A, 11B, 11C and 11D** show that the Rep/Cap promoter position affects genome packaging in two additional AAV vectors. (**FIGS. 11A and 11B**) Plots of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yield the distribution of sedimentation coefficients for the single stranded AAV5 Factor IX vector (AAV5 hFIX16) containing a cap5 sequence produced with a helper plasmid having an endogenous p5 promoter ("WT Rep," **FIG. 11B**) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," **FIG. 11A**). (**FIGS. 11C-11D**) Plots of differential sedimentation coefficient distribution value, c(s), vs sedimentation coefficient in Svedberg units (S), yield the distribution of sedimentation coefficients for the single stranded AAV5hSMN vector (AAV5SMN) containing a cap5 sequence produced with a helper plasmid having an endogenous p5 promoter ("WT Rep," **FIG. 11D**) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," **FIG. 11C**).

[0042] **FIGS. 12A and 12B** reveal that Southern blot analysis correlates with AUC analysis but misses some fragmented genomes detectable by AUC. (**FIG. 12A**) Southern blot analysis of vector DNA from AAV5SMN preparations made with the pHLP helper plasmid (lane 2) or the WT Rep plasmid (lane 1). 4.6 and 2.4 kb size standards are provided as labeled. (**FIG. 12B**) Southern blot analysis of vector DNA from AAV5FIX preparations made with the pHLP helper plasmid (lane 1) or the WT Rep plasmid (lane 2). 4.3, 3.0, and 1.9 kb size standards are provided as labeled.

[0043] **FIG. 13** provides a map of the AAV5 Factor IX vector indicating the positions of the hFIX transgene, ITR, Rep origin, and *AmpR* marker gene, among other features. Note that the *AmpR* marker is upstream of the ITR, enhancer, and promoter region.

[0044] **FIGS. 14A and 14B** show that WT Rep vector genomes, unlike pHLP Rep vector genomes, package sequences upstream of the 5' ITR in the AAV5 Factor IX vector. (**FIG. 14A**) Southern blot analysis using an hFIX transgene-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes. (**FIG. 14B**) Southern blot analysis using a Rep ori/*AmpR*-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes.

[0045] **FIGS. 15A and 15B** show the fragmentation of oversized AAV vector genomes, as demonstrated by AUC analysis. (**FIG. 15A**) Plot of concentration, C(S), versus sedimentation coefficient (S) generated by AUC for an AAV vector with an oversized genome. This genome contains a full-length chicken β-actin (CBA) promoter driving expression of β-phosphodiesterase (ssAAV2/5CBA-βPDE). Peaks for detected species are labeled by observed sedimentation coefficient (S) and relative abundance values (%). (**FIG. 15B**) Plot of concentration, C(S), versus sedimentation coefficient (S) generated by AUC for an AAV vector with a truncated genome. This genome contains a CBA promoter with a reduced-size intron driving expression of β-phosphodiesterase (AAV5 minCBAPDE6B). Peaks for detected species are labeled by observed sedimentation coefficient (S) and relative abundance values (%).

[0046] **FIG. 16** shows the AUC profiles of pure populations of adenovirus capsids. The sedimentation coefficient (S) and interference values are given for each peak.

DETAILED DESCRIPTION

[0047] The present invention provides methods of characterizing preparations of viral particles using analytical ultracentrifugation. By subjecting preparations to analytical ultracentrifugation (AUC) under boundary sedimentation velocity conditions, the sedimentation of viral particles can be monitored at time intervals (*e.g.*, one or more times). The differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S) is then plotted and the area under each peak in the C(s) distribution is integrated to determine the relative concentration of each peak. Each peak represents a species of viral particle reflective of its molecular weight. The species that can be detected by these methods include, but are not limited to, recombinant adeno-associated viral (rAAV) particles, recombinant adenoviral (rAd) particles, recombinant lentiviral particles, and recombinant herpes simplex viral (rHSV) particles. To use rAAV particles as an illustrative example, these methods allow the detection of rAAV species including rAAV capsid particles comprising intact rAAV genomes (*e.g.*, full capsids), empty viral capsids wherein no rAAV genomes have been encapsidated into viral capsids, and rAAV particle variants in which variant rAAV genomes are encapsidated in viral capsids (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates, and the like). These methods can be applied to preparations of viral particles regardless of nucleotide sequence of the viral genome or, in the case of recombinant viral

particles, the serotype of the recombinant viral capsid. These methods can be applied to rAAV, rAd, recombinant lentivirus and rHSV viral particles.

[0048] In some aspects, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles by subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times). By plotting the differential sedimentation coefficient distribution value C(S) versus the sedimentation coefficient in Svedberg units (S), species of recombinant viral particles in the preparation can be identified by presence of peaks on the plot corresponding to an S value. The genome size of a particular species of recombinant viral particles can be calculated, for example, by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of different known size. The vector genomes that can be assessed by these methods include, but are not limited to, recombinant viral capsid particles comprising intact recombinant viral genomes (*e.g.*, full capsids), empty viral capsids wherein no recombinant viral genomes have been encapsidated into viral capsids, and recombinant viral particle variants in which variant recombinant viral genomes (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates and the like) are encapsidated in viral capsids. In some embodiments, the viral particles are rAAV, rAd, recombinant lentivirus or rHSV viral particles.

[0049] In some embodiments the invention provides methods of determining the heterogeneity of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) in a preparation of recombinant viral particles by AUC under boundary sedimentation velocity conditions wherein the presence of peaks in a plot of C(S) v. S, in addition to the peak representing capsids comprising an intact viral genome, indicates heterogeneity of recombinant viral particles in the preparation. In some embodiments, the relative amounts of each recombinant viral species in the preparation are calculated by integrating the area for each peak in the plot.

[0050] In some embodiments of the invention, AUC is used to determine the presence of empty capsids and/or recombinant viral particle variants in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles), wherein the presence of peak that corresponds to the S value of empty capsid particles and/or recombinant viral particle variants in a

plot of C(S) vs. S indicates the presence of empty capsid particles and/or recombinant viral particle variants. In some embodiments, the relative amount of empty capsids and/or recombinant viral particle variants in a preparation of recombinant viral particles is determined by integrating the area under each peak in a plot of C(S) versus S and comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles and/or recombinant viral particle variants to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value corresponding to empty capsid particles and/or recombinant viral particle variants is compared to the total amount of all recombinant viral particles in the preparation by integrating and summing and the area under all the peaks of the plot.

[0051] In some embodiments, the invention provides methods of monitoring the removal of empty capsids and/or recombinant viral particle variants during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) by using AUC. Samples of the recombinant viral particles from the preparation following one or more steps in the purification process are analyzed for the relative amount of empty capsids and/or recombinant viral particle variants wherein a decrease in the relative amount of empty capsids and/or recombinant viral particle variants to full capsid particles indicates removal of empty capsids and/or recombinant viral particle variants from the preparation of recombinant viral particles.

[0052] In some embodiments, the invention provides methods of evaluating processes for the production of recombinant viral particles (*e.g.*, rAAV, rAd, lentivirus or rHSV particles) by AUC. The preparation of recombinant viral particles is analyzed for the presence of intact full viral capsid particles, empty particles and/or recombinant viral particle variants. An increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral particle variants (*e.g.*, particles containing AAV-encapsidated DNA impurities, truncated viral genomes, aggregates, and the like) compared to a reference preparation of recombinant viral particles (*e.g.*, a standard recombinant viral preparation process) indicates an improvement in the production of recombinant viral particles.

I. General Techniques

[0053] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, *et al.* eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Ausubel *et al.*, eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway *et al.*, 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita *et al.*, eds., J.B. Lippincott Company, 2011).

II. Definitions

[0054] A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

[0055] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term

includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate- phosphodiester oligomer. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0056] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0057] A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs).

[0058] A "recombinant AAV vector (recombinant adeno-associated viral vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one AAV inverted terminal repeat sequences (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (*i.e.* AAV Rep and Cap proteins). When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.,* in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an AAV particle. A recombinant viral vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (recombinant viral particle)".

[0059] An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

[0060] A "recombinant adenoviral vector" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of adenovirus origin) that are flanked by at least one adenovirus inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that is expressing essential adenovirus genes deleted from the recombinant viral genome (*e.g.*, E1 genes, E2 genes, E4 genes, etc.). When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.,* in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of adenovirus packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an adenovirus

particle. A recombinant viral vector can be packaged into an adenovirus virus capsid to generate a "recombinant adenoviral particle."

[0061] A "recombinant lentivirus vector" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentivirus origin) that are flanked by at least one lentivirus terminal repeat sequences (LTRs). In some embodiments, the recombinant nucleic acid is flanked by two lentiviral terminal repeat sequences (LTRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. A recombinant lentiviral vector can be packaged into a lentivirus capsid to generate a "recombinant lentiviral particle."

[0062] A "recombinant herpes simplex vector (recombinant HSV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of HSV origin) that are flanked by HSV terminal repeat sequences. Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. When a recombinant viral vector is incorporated into a larger polynucleotide (*e.g.,* in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of HSV packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an HSV particle. A recombinant viral vector can be packaged into an HSV capsid to generate a "recombinant herpes simplex viral particle."

[0063] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (*e.g.*, a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0064] The term "transgene" refers to a polynucleotide that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

[0065] The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant viral DNA genome or RNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark *et al.* (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk *et al.* (2002) *Mol. Ther.*, 6:272-278.

[0066] The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant viral vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example with AAV, in McLaughlin *et al.* (1988) *J. Virol.*, 62:1963-1973.

[0067] The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant viral vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example regarding AAV, in Xiao *et al.* (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher *et al.* (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0068] An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0069] An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several

shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0070] A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins.

[0071] "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0072] A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A helper virus provides "helper functions" which allow for the replication of AAV. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Examples of adenovirus helper functions for the replication of AAV include E1A functions, E1B functions, E2A functions, VA functions and E4orf6 functions. Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

[0073] A preparation of rAAV is said to be "substantially free" of helper virus if the ratio of infectious AAV particles to infectious helper virus particles is at least about 10^2:l; at least about 10^4:l, at least about 10^6:l; or at least about 10^8:l or more. In some embodiments, preparations are also free of equivalent amounts of helper virus proteins (*i.e.*, proteins as would be present as a result of such a level of helper virus if the helper virus particle impurities noted above were present in disrupted form). Viral and/or cellular protein contamination can generally be observed

as the presence of Coomassie staining bands on SDS gels (*e.g.*, the appearance of bands other than those corresponding to the AAV capsid proteins VPl, VP2 and VP3).

[0074] As used herein, "differential coefficient distribution value" or "C(S)" is a variant of the distribution of Lamm equation solutions to describe distributions of sedimenting particles; for example during ultracentrifugation.

[0075] As used herein, "Svedberg units" refers to a unit for sedimentation rate. The sedimentation rate for a particle of a given size and shape measures how fast the particle sediments. One Svedberg unit is equivalent to 10^{-13} seconds. For example, Svedberg units are often used to reflect the rate at which a molecule travels under the centrifugal force of a centrifuge.

[0076] As used herein, "sedimentation velocity conditions" or "boundary sedimentation velocity conditions" may refer to any experimental conditions under which a sample solution is subjected to sedimentation velocity analysis. Sedimentation velocity allows the study of particles over a wide range of pH and ionic strength conditions and at temperatures 4 to 40 °C. The rate at which the sedimentation boundary moves is a measure of the sedimentation coefficient of the sedimenting species. The sedimentation coefficient depends on the molecular weight (larger particles sediment faster) and also on molecular shape. The minimum width of the sedimentation boundary is related to the diffusion coefficient of the molecule; the presence of multiple species with similar sedimentation coefficients will cause the boundary to be broader than expected on the basis of diffusion alone. Sedimentation velocity conditions may include without limitation any conditions related to the rotor speed, distance between sample and rotor center, temperature, solvent, sample, buffer, ultracentrifugation time, time interval for detection, sector and optical window characteristics, AUC instrumentation (including ultracentrifuge and detection apparatus), equilibrium dialysis of reference solvent, and data analysis algorithms.

[0077] As used herein, the term "analytical density gradient sedimentation equilibrium" relates to methods for measuring the buoyant density of a particle, or using differences in buoyant density to separate different species of particles. These methods may use, for example, AUC sedimentation equilibrium techniques. In these methods, a particle solution (*e.g.,* without limitation, a solution of a polypeptide, polynucleotide, or viral capsids) may be subjected to

ultracentrifugation in a gradient solvate, such as a cesium chloride or cesium sulfate gradient, until equilibrium with the solvate is attained. At equilibrium, the particle solution will concentrate, or band, at the position in the gradient where the density of the particle is equal to that of the solvate. The position of bands may be used to calculate particle density, or a band may be extracted to isolate a single species of particle.

[0078] As used herein, the "SEDFIT algorithm" is an algorithm that allows one to analyze hydrodynamic data such as sedimentation velocity (Schuck (2000) *Biophys. J.*, 78:1606-19). In the SEDFIT algorithm, a grid of sedimentation coefficients across an expected range is created. Sedimentation boundaries are simulated using solutions to the Lamm equation for each sedimentation coefficient, assuming constant particle shape and solvent frictional ratio.

[0079] As used herein, the term "F statistic" or "F ratio" refers to the confidence level. This parameter controls the amount of regularization used. It has a different meaning for different ranges: From 0 to 0.5, no regularization is used. Values from 0.5 to 0.999 correspond to probabilities P (confidence levels). From these P-values, the desired chi-square increase allowed for the parsimony constraint of the regularization is calculated with F-statistics. A value of 0.51 will cause very little regularization; values of 0.68 to 0.90 would correspond to commonly used confidence levels (usually, with 50 scans or more the chi-square increase corresponding to a probability of 0.7 is of the order of 0.1%), while values close to 0.99 would cause very high regularization. The relationship of these values with probabilities can be examined using the F-statistics calculator. If numbers > 1 are entered, they are taken directly as chi-square ratios (as there are no probabilities > 1). For example, a value of 1.1 will result in regularization with 10% chi-square increase.

[0080] To "reduce" is to decrease, reduce or arrest an activity, function, and/or amount as compared to a reference. In certain embodiments, by "reduce" is meant the ability to cause an overall decrease of 20% or greater. In another embodiment, by "reduce" is meant the ability to cause an overall decrease of 50% or greater. In yet another embodiment, by "reduce" is meant the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater.

[0081] A "reference" as used herein, refers to any sample, standard, or level that is used for comparison purposes. For example, when measuring absorbance or refraction of AAV in an

aqueous solution, the absorbance or refraction of the solution is compared to the absorbance or refraction of the aqueous solution without AAV (*i.e.* a reference solution). In other examples, a reference may refer to a standard procedure known in the art. For example, when analyzing a procedure for improved quality of AAV production (*e.g.*, homogeneity), the AAV produced by the candidate procedure is compared to procedures known in the art (*i.e.* reference procedures).

[0082] An "isolated" molecule (*e.g.*, nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment. Thus, for example, isolated rAAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like, as defined below.

[0083] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X."

[0084] As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise. For example, the phrase "a rAAV particle" includes one or more rAAV particles.

[0085] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Analytical ultracentrifugation

[0086] Analytical ultracentrifugation is a means to evaluate the molecular weight and the hydrodynamic and thermodynamic properties of a protein or other macromolecule. Heterogeneity of a protein or macromolecule by sedimentation velocity over a range of conditions including concentration, temperature, ionic strength, and pH. For example, a protein may be analyzed in a

clinically relevant formulation. Use of analytical ultracentrifugation to characterize adenovirus preparations is provided by Berkowitz, SA & Philo JS, (2007) *Anal. Biochem.*, 362:16-37.

[0087] In certain aspects, the present invention provides methods of characterizing preparations of viral particles using analytical ultracentrifugation (AUC). For example, in some embodiments, the invention provides methods to assess vector genome integrity of recombinant adeno-associated viral (rAAV) particles in preparations of rAAV particles using AUC to distinguish viral particles with full, intact genomes, empty viral capsids and viral particles with variant (*e.g.,* truncated, aggregates, impurities and the like) viral genomes. In others embodiments, these methods may be applied in a similar way to analyze adenovirus, lentivirus, and herpes simplex virus (HSV) particles. AUC analysis refers to quantitative methods for characterizing the biophysical properties of particles (*e.g.,* polypeptides, polynucleotides, and viral capsids) by measuring their migration through a solvent in a centrifugal field. AUC analysis has been well characterized over many decades and is highly versatile. Because AUC analysis relies upon first-principle hydrodynamic and thermodynamic information, AUC may be applied to determine the biophysical properties of many types of particles across a wide range of particle concentrations and sizes. AUC analysis typically encompasses two basic types of experiment: sedimentation velocity and sedimentation equilibrium. Sedimentation equilibrium analysis yields thermodynamic properties of particles that may be used to measure characteristics such as stoichiometry and association constants. Sedimentation velocity yields hydrodynamic properties of particles that may be used to measure characteristics such as size, shape, and concentration. A feature of AUC analysis of viral preparations is that the same assay conditions may be used to analyze different preparations of viral particles regardless of nucleotide sequence of the viral genome or serotype of the capsid.

[0088] Certain aspects of the present disclosure relate to the use of sedimentation velocity analysis to characterize viral capsid properties. In some embodiments, sedimentation velocity analysis uses an ultracentrifuge velocity cell with two sectors in dialysis equilibrium (one for an experimental sample and one for a solvent-only reference sample), each containing two optical windows that allow light to pass through the compartment. Ultracentrifugation applies an angular velocity to the cell and leads to rapid sedimentation of the solute particles towards the bottom of the sector. As sedimentation occurs, solute is depleted near the meniscus at the top of the cell, creating a sedimenting boundary between the depleted region and the sedimenting solute. The

rate of movement or migration of the sedimenting boundary is measured by taking measurements that compare the properties of the sample and reference sectors at specific time intervals (for sedimentation velocity, these intervals are typically on the order of minutes). If multiple species of solute are present, this may lead to the formation of multiple sedimenting boundaries, each corresponding to a resolvable species.

[0089] Several methods for optically detecting a sedimenting boundary and measuring its rate of movement or migration are known in the art (for reference, see Cole *et al.* (2008) *Methods Cell Biol.*, 84:143-79). In some embodiments, the reference and sample sectors may be assayed using absorbance detection. In this detection method, the absorbance at a particular wavelength may be measured for the sample and reference sectors at different radial positions within each sector. Alternatively, the time course of absorbance at a single radial position may be measured. Beer's Law provides a mathematical relationship between absorbance and a solute's extinction coefficient.

[0090] In some embodiments, the reference and sample sectors may be assayed using interference detection (*e.g.*, Rayleigh interference detection). In the Rayleigh interference detection method, the interference optical system contains two parallel slits. A single, coherent beam of light is split such that it passes through both windows, and then the two beams are re-merged. When these two light waves are merged, they form an interference pattern of alternating light and dark fringes. If the sample and reference samples were to have an identical refractive index, the resulting interference fringes would be perfectly straight. Increasing the concentration of solute increases the solution's refractive index, thereby retarding the sample light beam and causing a vertical fringe shift. By measuring this fringe shift, one may measure the concentration of solute in the sample. Unlike absorbance detection, which measures absolute values for the sample and reference, interference detection measures a relative difference between the sample and reference. However, interference detection yields integrated peaks that are directly proportional to concentration, and it may be used for types of samples that do not absorb significantly. For a reference on using Rayleigh interference optics with AUC, see Furst (1997) *Eur. Biophys. J.* 35:307-10.

[0091] Measurement of the rate at which the sedimentation boundary moves may be used to derive many physical properties of solute particles. The rate of the boundary movement

determines the sedimentation coefficient, which is based on the mass and shape (frictional coefficient) of the particle. The sedimentation coefficient of a particle, *s*, refers to the ratio of its velocity to the acceleration applied to it by a centrifugal field. Sedimentation coefficients are expressed in Svedberg units, S (one Svedberg unit is equivalent to 10^{-13} seconds). The sedimentation coefficient of a particle or solution of particles depends upon its properties, for example molecular weight (corrected for buoyancy), and the properties of the solvent.

[0092] The change in the concentration boundary of a solute over time during ultracentrifugation may be determined using the Lamm equation (Schuck (2000) *Biophys. J.*, 78:1606-19). Briefly, the Lamm equation calculates the change in the concentration boundary of a solute over time in response to the competing forces of sedimentation (which concentrates the solute) and diffusion (which disperses the solute), taking into account the sector-shaped cell and the centrifugal field generated by the rotor. The Lamm equation may be expressed as:

Equation 1: $\partial c/\partial t=D [(\partial^2 c/\partial r^2)+1/r(\partial c/\partial r)]-s\omega^2 [r(\partial c/\partial r)+2c]$

where c is the solute concentration, D represents the solute diffusion constant, s represents the sedimentation coefficient, ω represents the angular velocity of the rotor, r is the radius, and t is time.

[0093] By fitting raw AUC data to solutions of the Lamm equation, it is possible to determine solute characteristics such as the sedimentation coefficient and the change in concentration distribution. For example, experimentally determined values for the rate of change of a sedimenting boundary may be modeled using the Lamm equation to derive the sedimentation coefficient, molecular mass, or concentration of the solute forming the boundary. Several programs known in the art, such as SEDFIT (Schuck (2000) *Biophys. J.*, 78:1606-19), may be used to model the Lamm equation to AUC data. These programs are also able to apply the Lamm equation to solutions containing multiple solutes or multiple sedimenting boundaries.

[0094] One example of a suitable program for the determination of solute characteristics is the SEDFIT algorithm. In some embodiments, the SEDFIT algorithm may be used to calculate a differential coefficient distribution value, or C(S), using AUC data from a solution containing a mixture of particle species (for reference, see Schuck (2000) *Biophys. J.*, 78:1606-19). In the SEDFIT algorithm, a grid of sedimentation coefficients across an expected range is created.

Sedimentation boundaries are simulated using solutions to the Lamm equation for each sedimentation coefficient, assuming constant particle shape and solvent frictional ratio. Actual AUC data are then fit to these Lamm solutions to derive the differential coefficient distribution value, or C(S). Many other programs useful for analyzing AUC data may be found in Cole and Hansen (1999) *J. Biomol. Tech.* 10:163-76.

[0095] In some embodiments of the invention, recombinant viral particles are highly purified, suitably buffered, and concentrated. In some embodiments, the viral particles are concentrated to at least about any of 1 x 10^7 vg/mL, 2 x 10^7 vg/mL, 3 x 10^7 vg/mL, 4 x 10^7 vg/mL, 5 x 10^7 vg/mL, 6 x 10^7 vg/mL, 7 x 10^7 vg/mL, 8 x 10^7 vg/mL, 9 x 10^7 vg/mL, 1 x 10^8 vg/mL, 2 x 10^8 vg/mL, 3 x 10^8 vg/mL, 4 x 10^8 vg/mL, 5 x 10^8 vg/mL, 6 x 10^8 vg/mL, 7 x 10^8 vg/mL, 8 x 10^8 vg/mL, 9 x 10^8 vg/mL, 1 x 10^9 vg/mL, 2 x 10^9 vg/mL, 3 x 10^9 vg/mL, 4 x 10^9 vg/mL, 5 x 10^9 vg/mL, 6 x 10^9 vg/mL, 7 x 10^9 vg/mL, 8 x 10^9 vg/mL, 9 x 10^9 vg/mL, 1 x 10^{10} vg/mL, 2 x 10^{10} vg/mL, 3 x 10^{10} vg/mL, 4 x 10^{10} vg/mL, 5 x 10^{10} vg/mL, 6 x 10^{10} vg/mL, 7 x 10^{10} vg/mL, 8 x 10^{10} vg/mL, 9 x 10^{10} vg/mL, 1 x 10^{11} vg/mL, 2 x 10^{11} vg/mL, 3 x 10^{11} vg/mL, 4 x 10^{11} vg/mL, 5 x 10^{11} vg/mL, 6 x 10^{11} vg/mL, 7 x 10^{11} vg/mL, 8 x 10^{11} vg/mL, 9 x 10^{11} vg/mL, 1 x 10^{12} vg/mL, 2 x 10^{12} vg/mL, 3 x 10^{12} vg/mL, 4 x 10^{12} vg/mL, 5 x 10^{12} vg/mL, 6 x 10^{12} vg/mL, 7 x 10^{12} vg/mL, 8 x 10^{12} vg/mL, 9 x 10^{12} vg/mL, 1 x 10^{13} vg/mL, 2 x 10^{13} vg/mL, 3 x 10^{13} vg/mL, 4 x 10^{13} vg/mL, 5 x 10^{13} vg/mL, 6 x 10^{13} vg/mL, 7 x 10^{13} vg/mL, 8 x 10^{13} vg/mL, 9 x 10^{13} vg/mL. In some embodiments, the viral particles are concentrated to of about 1 x 10^7 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{11} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^{12} vg/mL to about 1 x 10^{13} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^{11} vg/mL to about 1 x 10^{12} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^{10} vg/mL to about 1 x 10^{11} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^9 vg/mL to about 1 x 10^{10} vg/mL, about 1 x 10^7 vg/mL to about 1 x 10^9 vg/mL, about 1 x 10^8 vg/mL to about 1 x 10^9 vg/mL, or about 1 x 10^7 vg/mL to about 1 x 10^8 vg/mL.

[0096] In some embodiments, viral particles are generated in a suitable host cells and purified. In some embodiments, the viral particles are purified by affinity chromatography. Methods to

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purify viral particles (*e.g.*, AAV particles, adenovirus particles, lentivirus particles, HSV particles) are known in the art. For example, by use of an antibody of a viral capsid protein or binding ligand of a viral capsid protein immobilized on a chromatography media. Examples of viral capsid affinity chromatographies include but are not limited to AVB affinity chromatography for AAV (GE Healthcare), metal affinity chromatography for adenovirus and HSV, and heparin affinity chromatography for AAV and lentivirus, and the like. Methods to purify adenovirus particles are found, for example, in Bo, H *et al.*, (2014) *Eur. J. Pharm. Sci.* 67C:119-125. Methods to purify lentivirus particles are found, for example, in Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for example, in Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79.

[0097] In some embodiments, the recombinant viral particles are formulated in a pharmaceutical composition. In related embodiments, the pharmaceutical composition contains a buffer having physiological pH and/or physiological osmolality. A nonlimiting example of a pharmaceutical formulation is phosphate buffered saline (PBS) and in some embodiments, the PBS can be at physiological osmolality (*e.g.*, about pH 7.2 and about 300 mOsm/L). In some embodiments, sample adjustments are made to target concentration by optical density measurement at 260nm from 0.1 to 1.0. In some examples, this concentration results in reproducible and consistent AUC data. In some examples, concentration of viral particles is adjusted either by direct dilution with PBS or further concentration; for example, by using a centrifugal filter device.

[0098] In some embodiments of the invention, sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis is performed using an analytical ultracentrifuge that is capable of characterizing a sample in its native state under biologically relevant solution conditions (*e.g.*, ProteomeLab™ XL-I (Beckman Coulter)). When using the ProteomeLab™ XL-1, sample is loaded into the sample sector of a two sector velocity cell, a vehicle control (*e.g.*, PBS without recombinant viral) is loaded into the corresponding reference sector. The sample is placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of about 20 ^{o}C and full vacuum are maintained for about one hour. In an exemplary embodiment, sedimentation velocity centrifugation is performed at about 20,000 RPM, about 20 °C, and about 0.003 cm radial step setting, with no delay and with no replicates. As noted below, different parameters may be used for centrifugation. In some embodiments, absorbance (260 nm) and/or

interference optics (*e.g.*, Rayleigh interference optics) are used to simultaneously record radial concentration as a function of time until the smallest sedimenting component clears the optical window. In some embodiments, the radial concentration is recorded until the sedimenting species with the lowest density clears the sector. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge. A sector may be a portion of an ultracentrifuge; for example an ultracentrifuge velocity cell. In some embodiments, a sector may be a portion of an ultracentrifuge where samples are detected. In some embodiments, the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell. In some embodiments, is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell. In some embodiments, sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears the optical window. In some embodiments, the radial concentration is recorded for at least about any of 0.5 hours, 0.75 hours, 1.0 hours, 1.5 hours, 2.0 hours, 3.0 hours, 4.0 hours, or 5.0 hours. In some embodiments, the radial concentration is recorded for between any of about 0.5 hours to about 0.75 hours, about 0.75 hours to about 1.0 hours, about 1.0 hours to about 1.5 hours, about 1.5 hours to about 2.0 hours, about 2 hours to about 3 hours, about 3 hours to about 4 hours, about 4 hours to about 5 hours. In some embodiments, the radial concentration is recorded for about 1.2 hours. Optimizing runs conditions may include, for example, continuing the run until all of the sedimenting species are fully sedimented to the bottom of the sector, with the temperature held constant at 20 ºC and a speed between 18,000 rpm and 20,000 rpm. As noted below, other temperatures and speeds may be used.

[0099] The percent full capsid is determined by analyzing a multiple of scans (*e.g.*, 75) from each detection method using the SEDFIT continuous size C(S) distribution model. Second (2nd) derivative regularization is applied to the fitting. In some embodiments, the confidence level of F statistic is about 0.68. In some embodiments, the confidence level of F statistic is more than about any of 0.68, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95 or 0.99. In some embodiments, the confidence level of F statistic is about 0.68 to about 0.90. In some embodiments, the confidence level of F statistic is about 0.68 to about 0.99. In some embodiments, the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software. In some embodiments, the resolution is about any of 200S, 300S,

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400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the resolution is between any of about 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, the resolution is about 200S. In some embodiments, the Smax is about any of 100S, 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the Smax is between any of about 100S to about 1000S, 100S to about 900S, 100S to about 800S, 100S to about 700S, 100S to about 600S, 100S to about 500S, 100S to about 400S, 100S to about 300S, 100S to about 200S, 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, Smax is about 200S to about 5000S. In some embodiments, wherein Smax is about 200S. In some embodiments, the frictional ratio is left to float to a value determined by centrifugation software. In some embodiments, the frictional ratio is about 1.0. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied. In some embodiments, the meniscus position is allowed to float, letting the software choose the optimal position. In some embodiments, the frictional ratio is allowed to float, letting the software choose the optimal position. The model fits the data to the Lamm equation, and the resulting size distribution is a "distribution of sedimentation coefficients" that looks like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) are determined for each component in the distribution. In some

embodiments, multiple AUC runs are independent assays, and each analysis the following attributes are monitored to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\ nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans.

[0100] In some embodiments of the invention, extinction coefficients are used to calculate molar concentration and the actual percent value of the intact vector peak from absorbance data. Molar absorbance extinction coefficients for both empty capsids ($\epsilon_{260}/_{capsid}$=3.72e6) and intact vector ($\epsilon_{260}/_{vector}$=3.00e7) can be calculated based on published formulae (Sommer *et al.* (2003) *Mol Ther.*, 7:122-8). Extinction coefficients are available for empty capsid and intact vector peaks. The C(S) values can be determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.,* 78:1606-19. Molar concentration of both intact vector and empty capsid can be calculated using Beer's Law and the percentage of full capsid are calculated from these values. In some embodiments, values are reported in terms of the percentage of full capsid.

[0101] In some embodiments, it is not possible to determine empirically the extinction coefficient of particular species of recombinant viral particles (*e.g.*, viral particles with fragmented genomes of unknown size and sequence). A relationship between S value and genome size may be established by analyzing recombinant viral vector preps with encapsidated viral genomes of known nucleotide size and a corresponding S value are determined as described herein. The calculated S values can be plotted to generate a standard curve to which recombinant viral species of unknown molecular weight or genome size can be compared to determine the molecular weight of the unknown species.

[0102] In some aspects, the invention provides methods of characterizing a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle. In some embodiments, the species of recombinant viral particle identified by the methods of the invention include, but are

not limited to; full recombinant viral particles comprising intact recombinant viral genomes, empty recombinant viral capsid particles, and recombinant viral particles comprising variant recombinant viral genomes. In some embodiments the variant genomes are smaller than the intact recombinant viral genome (*e.g.*, truncated genomes). In some embodiments, the variant genomes are larger than the intact recombinant viral genome (*e.g.*, aggregates, recombinants, *etc.*). In some embodiments, the invention provides methods to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of different known size. In some embodiments, the methods further comprise integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles. In some embodiments, the sedimentation of recombinant viral particles is monitored at one time interval. In some embodiments, the sedimentation of recombinant viral particles is monitored at more than one time interval.

[0103] In some embodiments of the invention, the sedimentation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) is monitored by measuring optical density or absorbance at about 260 nm. Means of measuring absorbance are known in the art. In some embodiments, an ultracentrifuge used for AUC is equipped with means for measuring absorbance. In other embodiments, the sedimentation of recombinant viral particles is monitored by interference. In some embodiments, the sedimentation of recombinant viral particles is monitored by Rayleigh interference. Means of measuring interference are known in the art (Furst (1997) *Eur. Biophys. J.* 35:307-10). In some embodiments, an ultracentrifuge used for AUC is equipped with means for measuring interference. In some embodiments, the sedimentation of recombinant viral particles is monitored by both absorbance and interference. In some embodiments, the absorbance and/or interference are measured using a reference standard. In some embodiments,

the reference standard matches the solution of the recombinant viral preparation with the exception that the recombinant viral is not present. For example, the recombinant viral preparation may comprise recombinant viral in a buffer such as phosphate buffered saline. In this example, the reference standard may be phosphate buffered saline without recombinant viral particles.

[0104] In some embodiments of the invention, the preparation of viral particles is in a pharmaceutical formulation. Such formulations are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580). Such pharmaceutical formulations can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical formulation may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. In some embodiments of the invention the pharmaceutical formulation comprises phosphate buffered saline.

[0105] In some embodiments of the invention, the sedimentation velocity of viral particles during ultracentrifugation is determined by monitoring the sedimentation of viral particles continuously during ultracentrifugation. It is within the purview of the skilled artisan to optimize the parameters of AUC for different types of viral particles. Without wishing to be bound to theory, a range of AUC settings that allows the analysis of both AAV and adenovirus particles should enable the analysis of other viral particles including lentivirus and HSV since the size of HSV and lentiviral particles is between that of AAV and adenovirus particles. In some embodiments, data acquisition for rAAV, rHSV, lentiviral, and/or rAd particles is performed with an AUC speed of between about 3,000 and about 20,000 rpm. In some embodiments, data analysis for rAAV, HSV, lentiviral, and/or adenoviral particles is performed with an S_{min} of about 1S and an S_{max} of about 1000S. In some embodiments, data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with a resolution of about 200S to about 1,000S. In some embodiments, the resolution is about any of 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the resolution is between any of about 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S,

200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, the resolution is about 200S. data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with an Smax of about any of 100S, 200S, 300S, 400S, 500S, 600S, 700S, 800S, 900S, or 1000S. In some embodiments, the Smax is between any of about 100S to about 1000S, 100S to about 900S, 100S to about 800S, 100S to about 700S, 100S to about 600S, 100S to about 500S, 100S to about 400S, 100S to about 300S, 100S to about 200S, 200S to about 1000S, 200S to about 900S, 200S to about 800S, 200S to about 700S, 200S to about 600S, 200S to about 500S, 200S to about 400S, 200S to about 300S, 300S to about 1000S, 300S to about 900S, 300S to about 800S, 300S to about 700S, 300S to about 600S, 300S to about 500S, 300S to about 400S, 400S to about 1000S, 400S to about 900S, 400S to about 800S, 400S to about 700S, 400S to about 600S, 400S to about 500S, 500S to about 1000S, 500S to about 900S, 500S to about 800S, 500S to about 700S, 500S to about 600S, 600S to about 1000S, 600S to about 900S, 600S to about 800S, 600S to about 700S, 700S to about 1000S, 700S to about 900S, 700S to about 800S, 800S to about 1000S, 800S to about 900S, or 900S to about 1000S. In some embodiments, Smax is about 200S to about 5000S. In some embodiments, wherein Smax is about 200S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied. In some embodiments, the meniscus position is allowed to float, letting the software choose the optimal position. In some embodiments, the frictional ratio is allowed to float, letting the software choose the optimal position. In some embodiments, data analysis for rAAV and/or adenoviral particles is held constant at 1. In some embodiments, data analysis for rAAV, HSV, lentiviral, and/or adenoviral particles is allowed to float by using the FIT command with a value optimized using non-linear regression.

[0106] With respect to recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles), in some embodiments, the sedimentation velocity of recombinant viral during ultracentrifugation is determined by monitoring (*e.g.*, scanning) the sedimentation of recombinant viral particles once in more than about every 15 seconds, 30 seconds, 45 seconds, 1 minute (60

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seconds), 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes. Scans may be continuously acquired without delay as quickly as the optical systems allow. Interference scans are rapid, and a single scan is complete in ~10-15 seconds, while absorbance scans require ~ 60 seconds. When dual detection is used the speed of scan acquisition for both are determined by the absorbance system. In some embodiments of the invention, more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 scans are used to monitor sedimentation of recombinant viral particles during ultracentrifugation. In some embodiments, a minimum of 30 scans is required for analysis, and scans are collected until the sedimentation process is complete. In some embodiments, the sedimentation process may typically be described by between 40 and 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 55 scans to about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 55 scans to about 60 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 60 scans to about 75 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on about 60 scans to about 70 scans. In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on multiple ultracentrifugations (runs). In some embodiments, the sedimentation velocity of recombinant viral particles is determined based on any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more ultracentrifugation runs. In some embodiments, the sedimentation velocities are used to determine C(S) values using the SEDFIT algorithm. In some embodiments, a second derivative regularization is applied to a fitting level with a confidence level of F statistic of about 0.68. In some embodiments, the following C(S) parameters are held constant: resolution 100S to about 200S, S min is about 1, S max is about 200S to 300S, and frictional ratio is about 1.0 to 1.2S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

[0107] In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at more than about any of 5,000 rpm; 10,000 rpm; 15,000 rpm; 20,000 rpm; 25,000 rpm; 30,000 rpm; 35,000 rpm; 40,000 rpm; 45,000 rpm; or 50,000 rpm. In some embodiments, the

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ultracentrifugation is run at between any of about 5,000 rpm and about 50,000 rpm; about 10,000 rpm and about 50,000 rpm; about 15,000 rpm and about 50,000 rpm; about 20,000 rpm and about 50,000 rpm; about 25,000 rpm and about 50,000 rpm; about 30,000 rpm and about 50,000 rpm; about 35,000 rpm and about 50,000 rpm; about 40,000 rpm and about 50,000 rpm; about 45,000 rpm and about 50,000 rpm; about 5,000 rpm and about 45,000 rpm; about 10,000 rpm and about 45,000 rpm; about 15,000 rpm and about 45,000 rpm; about 20,000 rpm and about 45,000 rpm; about 25,000 rpm and about 45,000 rpm; about 30,000 rpm and about 45,000 rpm; about 40,000 rpm and about 45,000 rpm; about 5,000 rpm and about 40,000 rpm; about 10,000 rpm and about 40,000 rpm; about 15,000 rpm and about 40,000 rpm; about 20,000 rpm and about 40,000 rpm; about 25,000 rpm and about 40,000 rpm; about 30,000 rpm and about 40,000 rpm; about 35,000 rpm and about 40,000 rpm; about 5,000 rpm and about 35,000 rpm; about 10,000 rpm and about 35,000 rpm; about 15,000 rpm and about 35,000 rpm; about 20,000 rpm and about 35,000 rpm; about 25,000 rpm and about 35,000 rpm; about 30,000 rpm and about 35,000 rpm; about 5,000 rpm and about 30,000 rpm; about 10,000 rpm and about 30,000 rpm; about 15,000 rpm and about 30,000 rpm; about 20,000 rpm and about 30,000 rpm; about 25,000 rpm and about 30,000 rpm; about 5,000 rpm and about 25,000 rpm; about 10,000 rpm and about 25,000 rpm; about 20,000 rpm and about 25,000 rpm; about 5,000 rpm and about 20,000 rpm; about 10,000 rpm and about 20,000 rpm; about 15,000 rpm and about 20,000 rpm; about 5,000 rpm and about 15,000 rpm; about 10,000 rpm and about 15,000 rpm; or about 5,000 rpm and about 10,000 rpm. In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 20,000 rpm. In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 15,000 rpm to about 20,000 rpm.

[0108] In some embodiments of the invention, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) by ultracentrifuging the preparation of recombinant viral particles at about or more than 4 °C, 10 °C, 15 °C, 20 °C, 25 °C, or 30 °C. In some embodiments, the ultracentrifugation is run as between any of about 4 °C and about 30 °C, about 4 °C and about 25 °C, about 4 °C and about 20 °C, about 4 °C and about 15 °C, about 4 °C and about 10 °C, about

10 °C and about 30 °C, about 10 °C and about 25 °C, about 10 °C and about 20 °C, about 10 °C and about 15 °C, about 15 °C and about 30 °C, about 15 °C and about 25 °C, about 15 °C and about 20 °C, about 20 °C and about 30 °C, or about 20 °C and about 25 °C. In some embodiments, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 20 °C. In some embodiments, the boundary sedimentation velocity of recombinant viral particles in a preparation of recombinant viral particles by ultracentrifuging the preparation of recombinant viral particles at about 15 °C to about 20 °C.

[0109] As disclosed herein, numerous types of recombinant viral particles may be analyzed by the methods of the present disclosure (*e.g.,* AAV, adenoviral, lentiviral, and/or HSV particles). Suitable ultracentrifugation conditions, analysis algorithms, and other parameters may be determined empirically through methods known in the art. Exemplary parameters for AAV, adenoviral, lentiviral, and HSV particles, along with guidance for the selection of specific parameter options, are provided without limitation in Table 1 below.

Table 1. Exemplary parameters for AAV, adenoviral, lentiviral, and HSV particles.

	AAV	**Ad**	**Lentivirus**	**HSV**
Exemplary buffers	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L	Phosphate based buffer at physiologic pH, and physiologic osmolality ~ 300 mOsM/L
Exemplary algorithms for determining C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)	Any algorithm using Lamm equation solutions; e.g., SEDFIT C(S)
Exemplary number of scans (minimum, maximum, ranges)	30-999 *excess scans can always be collected and then excluded from analysis (such as skip every other scan) -scans that occur after complete sedimentation of virus can be	30-999	30-999	30-999

	excluded			
Exemplary confidence level of the F statistic	F=0.68	F=0.68	F=0.68	F=0.68
Exemplary ranges for Smin	1-100S	1-100S	1-100S	1-100S
Exemplary ranges for Smax	100-1000S	100-5000S	100-5000S	100-5000S
Exemplary ranges for resolution	*Resolution depends on S Max 200S-1000S	200-5000S	200-5000S	200-5000S
Exemplary frictional ratio	Use the FIT command to determine frictional ratio. Since AAV is ~spherical, in embodiments, 1 may be used as the frictional ratio.	Use FIT command to determine frictional ratio or set at 1.	Use FIT command to determine frictional ratio	Use FIT command to determine frictional ratio
Exemplary ranges for AUC speed	10,000-20,000 rpm	3,000-10,000 rpm	3,000-10,000 rpm	3,000-10,000 rpm
Exemplary absorbances for monitoring sedimentation of viral particles	260nm 280 nm 230 nm	260nm 280 nm 230 nm	260nm 280 nm 230 nm	260nm 280 nm 230 nm
Exemplary methods for monitoring sedimentation of viral particles	IF Absorbance	IF Absorbance	IF Absorbance	IF Absorbance
Radial invariant (RI) and time invariant (TI) noise subtractions, alternative subtractions/calculations	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection	Ti and RI noise correction required for interference detection. May or may not be used with absorbance detection
Scanning frequency	*when using absorbance detection system, limited by speed of absorbance scan (~60 Seconds)-scan as fast as system	Scan with no delay through scan with 60 second delay	Scan with no delay through scan with 60 second delay	Scan with no delay through scan with 60 second delay

	allows with no delay IF only: collect every 10 -60 seconds (10-60 second delay)			
Temperature ranges	4°C -20°C	4°C -20°C	4°C -20°C	4°C -20°C

[0110] In some aspects, the invention provides methods to determine the presence of empty capsids in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peak that corresponds to the S value of empty capsid particles indicates that presence of empty capsid particles. In some embodiments, the invention provides methods of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value corresponding to empty capsid particles is compared to the total amount of all recombinant viral particles in the preparation by integrating all peaks on the plot of C(S) vs. S.

[0111] In some aspects, the invention provides methods to determine the presence of recombinant viral particle variants in a preparation of recombinant viral particles (*e.g.*, rAAV,

rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peak that corresponds to the S value that differs from the S value of recombinant viral capsid particles comprising a full intact recombinant viral genome indicates that presence of recombinant viral particle variants. In some embodiments, the invention provides methods of measuring the relative amount recombinant viral particle variants in a preparation of recombinant viral particles comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes. In some embodiments, the amount of recombinant viral particles having an S value that differs from the S value of recombinant viral capsid particles comprising a full intact recombinant viral genome is compared to the total amount of all recombinant viral particles in the preparation by integrating all peaks on the plot of C(S) vs. S. In some embodiments, the recombinant viral particle variants comprise recombinant viral genomes that are smaller (*e.g.*, truncated) or larger than the full length intact viral genome. Other viral-encapsidated DNA impurities can also be detected.

[0112] In some embodiments, the invention provides methods of monitoring the removal of empty capsids and/or recombinant viral particles with variant genomes during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids using AUC as described herein. A decrease in the relative amount of

empty capsids and/or recombinant viral particles comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

[0113] In some embodiments, the invention provides methods of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) comprising the steps of a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (*e.g.*, one or more times), b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant viral particles in the preparation. In some embodiments, the species of recombinant viral particle identified by the methods of the invention include, but are not limited to full recombinant viral particles comprising intact recombinant viral genomes, empty recombinant viral capsid particles, and recombinant viral particles comprising variant recombinant viral genomes. In some embodiments the variant genomes are smaller than the intact recombinant viral genome (*e.g.*, truncated genomes). In some embodiments, the variant genomes are larger than the intact recombinant viral genome (*e.g.*, aggregates, recombinants, *etc.*). In some embodiments the variant genomes include genomes that are smaller and larger than the intact recombinant viral genome.

[0114] In some embodiments, the invention provides methods of monitoring the heterogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles (*e.g.*, rAAV, rAd, lentiviral, or rHSV particles) the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the relative amount of full capsids comprising an intact recombinant viral genome, empty capsids and/or recombinant viral particles with variant genomes using AUC as described herein, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

[0115] In embodiments of the embodiments described above, the recombinant viral particles have been purified using one or more purification steps. Examples of purification steps include but are not limited to equilibrium centrifugation, anion exchange filtration, tangential flow

filtration (TFF), apatite chromatography, heat inactivation of helper virus, hydrophobic interaction chromatography, immunoaffinity chromatography, size exclusion chromatography (SEC), nanofiltration, cation exchange chromatography, and anion exchange chromatography.

[0116] In embodiments of the embodiments described above, the recombinant viral particles comprise a self-complementary AAV (scAAV) genome. In some embodiments, the recombinant AAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, a therapeutic transgene coding strand) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the therapeutic transgene) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide sequence along most or all of its length. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR. In some embodiments, the scAAV viral particles comprise a monomeric form of an scAAV genome. In some embodiments, the scAAV viral particles comprise the dimeric form of and scAAV genome. In some embodiments, AUC as described herein is used to detect the presence of rAAV particles comprising the monomeric form of an scAAV genome. In some embodiments, AUC as described herein is used to detect the presence of rAAV particles comprising the dimeric form of an scAAV genome. In some embodiments, the packaging of scAAV genomes into capsid is monitored by AUC described herein.

[0117] In embodiments of the embodiments described above, the rAAV particles comprise an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid (*e.g.*, a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAVrh8R, an AAV9 capsid (*e.g.*, a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV DJ capsid (*e.g.*, an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an

AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In embodiments of the above embodiments described above, the rAAV particles comprise at least one AAV1 ITR, AAV2 ITR, AAV3 ITR, AAV4 ITR, AAV5 ITR, AAV6 ITR, AAV7 ITR, AAV8 ITR, AAVrh8 ITR, AAV9 ITR, AAV10 ITR, AAVrh10 ITR, AAV11 ITR, AAV12 ITR, AAV DJ ITR, goat AAV ITR, bovine AAV ITR, or mouse AAV ITR. In some embodiments, the rAAV particles comprise ITRs from one AAV serotype and AAV capsid from another serotype. For example, the rAAV particles may comprise a therapeutic transgene flanked by at least one AAV2 ITR encapsidated into an AAV9 capsid. Such combinations may be referred to as pseudotyped rAAV particles.

IV. Viral particles

[0118] The methods disclosed herein may find use, *inter alia,* in characterizing species of interest in a variety of viral particles (*e.g.,* viral particles with a full genome, as compared to viral particles with truncated genomes and/or viral particles comprising DNA impurities).

[0119] In some embodiments, the viral particle is a recombinant AAV particle comprising a nucleic acid comprising a transgene flanked by one or two ITRs. The nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises the protein coding sequence(s) of interest (*e.g.*, a therapeutic transgene) operatively linked components in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette. The expression cassette is flanked on the 5' and 3' end by at least one functional AAV ITR sequences. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson *et al.*, *PNAS*, 2000, 97(7)3428-32; Passini *et al.*, *J. Virol.*, 2003, 77(12):7034-40; and Pechan *et al.*, *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (*e.g.*, as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV

serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao *et al.*, *PNAS*, 2002, 99(18): 11854-6; Gao *et al.*, *PNAS*, 2003, 100(10):6081-6; and Bossis *et al.*, *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid, or the like. In some embodiments, the nucleic acid in the AAV comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, AAV12 or the like. In further embodiments, the rAAV particle comprises capsid proteins of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAV11, AAV12 or the like. In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, *et al. J. Virol.* 2004, 78(12):6381).

[0120] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle can comprise AAV9 capsid proteins and at least one AAV2 ITR or it can comprise AAV2 capsid proteins and at least one AAV9 ITR. In yet another example, a rAAV particle can comprise capsid proteins from both AAV9 and AAV2, and further comprise at least one AAV2 ITR. Any combination of AAV serotypes for production of a rAAV particle is provided herein as if each combination had been expressly stated herein.

[0121] In some embodiments, the AAV comprises at least one AAV1 ITR and capsid protein from any of AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV2 ITR and capsid protein from any of AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV3 ITR and capsid protein from any of AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV

comprises at least one AAV4 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV5 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV6 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV7 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV8 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV9 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh.8, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAVrh8 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh10, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAVrh10 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV11, and/or AAV12. In some embodiments, the AAV comprises at least one AAV11 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAV9, AAVrh10, and/or AAV12. In some embodiments, the AAV comprises at least one AAV12 ITR and capsid protein from any of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV rh8, AAV9, AAVrh10, and/or AAV11.

Self-complementary AAV viral genomes

[0122] In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome. AAV viral particles with self-complementing genomes and methods of use of self-complementing AAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,765,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., *et al.*, (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (*e.g.*, complementing coding and non-coding strands of a transgene). In some embodiments, the invention provides an AAV

viral particle comprising an AAV genome, wherein the rAAV genome comprises a first heterologous polynucleotide sequence (*e.g.*, a therapeutic transgene coding strand) and a second heterologous polynucleotide sequence (*e.g.*, the noncoding or antisense strand of the therapeutic transgene) wherein the first heterologous polynucleotide sequence can form intrastrand base pairs with the second polynucleotide sequence along most or all of its length. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a sequence that facilitates intrastrand basepairing; *e.g.*, a hairpin DNA structure. Hairpin structures are known in the art, for example in siRNA molecules. In some embodiments, the first heterologous polynucleotide sequence and a second heterologous polynucleotide sequence are linked by a mutated ITR (*e.g.*, the right ITR). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0123] In some embodiments, the viral particle is an adenoviral particle. In some embodiments, the adenoviral particle is a recombinant adenoviral particle, *e.g.,* a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of adenoviral origin) between two ITRs. In some embodiments, the adenoviral particle lacks or contains a defective copy of one or more E1 genes, which renders the adenovirus replication-defective. Adenoviruses include a linear, double-stranded DNA genome within a large (~950Å), non-enveloped icosahedral capsid. Adenoviruses have a large genome that can incorporate more than 30kb of heterologous sequence (*e.g.,* in place of the E1 and/or E3 region), making them uniquely suited for use with larger heterologous genes. They are also known to infect dividing and non-dividing cells and do not naturally integrate into the host genome (although hybrid variants may possess this ability). In some embodiments, the adenoviral vector may be a first generation adenoviral vector with a heterologous sequence in place of E1. In some embodiments, the adenoviral vector may be a second generation adenoviral vector with additional mutations or deletions in E2A, E2B, and/or E4. In some embodiments, the adenoviral vector may be a third generation or gutted adenoviral vector that lacks all viral coding genes, retaining only the ITRs

and packaging signal and requiring a helper adenovirus *in trans* for replication, and packaging. Adenoviral particles have been investigated for use as vectors for transient transfection of mammalian cells as well as gene therapy vectors. For further description, see, *e.g.,* Danthinne, X. and Imperiale, M.J. (2000) *Gene Ther.* 7:1707-14 and Tatsis, N. and Ertl, H.C. (2004) *Mol. Ther.* 10:616-29.

[0124] In some embodiments, the viral particle is a recombinant adenoviral particle comprising a nucleic acid comprising a transgene. Use of any adenovirus serotype is considered within the scope of the present invention. In some embodiments, the recombinant adenoviral vector is a vector derived from an adenovirus serotype, including without limitation, AdHu2, AdHu 3, AdHu4, AdHu5, AdHu7, AdHu11, AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, and porcine Ad type 3. The adenoviral particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise an adenoviral particle in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped recombinant adenoviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped recombinant adenoviral particles are derived from a foreign virus or from another adenovirus serotype. In some embodiments, the foreign viral capsid proteins are derived from, including without limitation, reovirus type 3. Examples of vector and capsid protein combinations used in pseudotyped adenovirus particles can be found in the following references (Tatsis, N. *et al.* (2004) *Mol. Ther.* 10(4):616-629 and Ahi, Y. *et al.* (2011) *Curr. Gene Ther.* 11(4):307-320). Different adenovirus serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes, include without limitation, lung (*e.g.* HuAd3), spleen and liver (*e.g.* HuAd37), smooth muscle, synoviocytes, dendritic cells, cardiovascular cells, tumor cell lines (*e.g.* HuAd11), and dendritic cells (*e.g.* HuAd5 pseudotyped with *reovirus type 3, HuAd30, or HuAd35*). For further description, see Ahi, Y. *et al.* (2011) *Curr. Gene Ther.* 11(4):307-320, Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40, and Tatsis, N. *et al.* (2004) *Mol. Ther.* 10(4):616-629.

[0125] In some embodiments, the viral particle is a lentiviral particle. In some embodiments, the lentiviral particle is a recombinant lentiviral particle, *e.g.,* a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentiviral origin) between

two LTRs. Lentiviruses are positive-sense, ssRNA retroviruses with a genome of approximately 10 kb. Lentiviruses are known to integrate into the genome of dividing and non-dividing cells. Lentiviral particles may be produced, for example, by transfecting multiple plasmids (typically the lentiviral genome and the genes required for replication and/or packaging are separated to prevent viral replication) into a packaging cell line, which packages the modified lentiviral genome into lentiviral particles. In some embodiments, a lentiviral particle may refer to a first generation vector that lacks the envelope protein. In some embodiments, a lentiviral particle may refer to a second generation vector that lacks all genes except the gag/pol and tat/rev regions. In some embodiments, a lentiviral particle may refer to a third generation vector that only contains the endogenous rev, gag, and pol genes and has a chimeric LTR for transduction without the tat gene (see Dull, T. *et al.* (1998) *J. Virol.* 72:8463-71). For further description, see Durand, S. and Cimarelli, A. (2011) *Viruses* 3:132-59.

[0126] In some embodiments, the viral particle is a recombinant lentiviral particle comprising a nucleic acid comprising a transgene. Use of any lentiviral vector is considered within the scope of the present invention. In some embodiments, the lentiviral vector is derived from a lentivirus including, without limitation, human immunodeficiency virus-1 (HIV-1), human immunodeficiency virus-2 (HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), Jembrana disease virus (JDV), visna virus (VV), and caprine arthritis encephalitis virus (CAEV). The lentiviral particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise a lentivirus vector in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped recombinant lentiviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped recombinant lentiviral particles are derived from a foreign virus. In some embodiments, the foreign viral capsid protein used in pseudotyped recombinant lentiviral particles is Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped recombinant lentiviral particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped recombinant lentiviral particles. In other embodiments, the foreign viral capsid proteins are derived from, including without limitation, Chandipura virus, Rabies virus, Mokola virus, Lymphocytic choriomeningitis virus (LCMV), Ross River virus (RRV), Sindbis virus, Semliki Forest virus (SFV), Venezuelan equine

encephalitis virus, Ebola virus Reston, Ebola virus Zaire, Marburg virus, Lassa virus, Avian leukosis virus (ALV), Jaagsiekte sheep retrovirus (JSRV), Moloney Murine leukemia virus (MLV), Gibbon ape leukemia virus (GALV), Feline endogenous retrovirus (RD114), Human T-lymphotropic virus 1 (HTLV-1), Human foamy virus, Maedi-visna virus (MVV), SARS-CoV, Sendai virus, Respiratory syncytia virus (RSV), Human parainfluenza virus type 3, Hepatitis C virus (HCV), Influenza virus, Fowl plague virus (FPV), or Autographa californica multiple nucleopolyhedro virus (AcMNPV). Examples of vector and capsid protein combinations used in pseudotyped Lentivirus particles can be found, for example, in Cronin, J. *et al.* (2005). *Curr. Gene Ther.* 5(4):387-398. Different pseudotyped recombinant lentiviral particles can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). For example, tissues targeted by specific pseudotyped recombinant lentiviral particles, include without limitation, liver (*e.g.* pseudotyped with a VSV-G, LCMV, RRV, or SeV F protein), lung (*e.g.* pseudotyped with an Ebola, Marburg, SeV F and HN, or JSRV protein), pancreatic islet cells (*e.g.* pseudotyped with an LCMV protein), central nervous system (*e.g.* pseudotyped with a VSV-G, LCMV, Rabies, or Mokola protein), retina (*e.g.* pseudotyped with a VSV-G or Mokola protein), monocytes or muscle (*e.g.* pseudotyped with a Mokola or Ebola protein), hematopoietic system (*e.g.* pseudotyped with an RD114 or GALV protein), or cancer cells (*e.g.* pseudotyped with a GALV or LCMV protein). For further description, see Cronin, J. *et al.* (2005). *Curr. Gene Ther.* 5(4):387-398 and Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40.

[0127] In some embodiments, the viral particle is a herpes simplex virus (HSV) particle. In some embodiments, the HSV particle is a rHSV particle, *e.g.,* a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of lentiviral origin) between two TRs. HSV is an enveloped, double-stranded DNA virus with a genome of approximately 152 kb. Advantageously, approximately half of its genes are nonessential and may be deleted to accommodate heterologous sequence. HSV particles infect non-dividing cells. In addition, they naturally establish latency in neurons, travel by retrograde transport, and can be transferred across synapses, making them advantageous for transfection of neurons and/or gene therapy approaches involving the nervous system. In some embodiments, the HSV particle may be replication-defective or replication-competent (*e.g.,* competent for a single replication cycle through

inactivation of one or more late genes). For further description, see Manservigi, R. *et al.* (2010) *Open Virol. J.* 4:123-56.

[0128] In some embodiments, the viral particle is a rHSV particle comprising a nucleic acid comprising a transgene. Use of any HSV vector is considered within the scope of the present invention. In some embodiments, the HSV vector is derived from a HSV serotype, including without limitation, HSV-1 and HSV-2. The HSV particle also comprises capsid proteins. In some embodiments, the recombinant viral particles comprise a HSV vector in combination with one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped rHSV particles. In some embodiments, foreign viral capsid proteins used in pseudotyped rHSV particles are derived from a foreign virus or from another HSV serotype. In some embodiments, the foreign viral capsid protein used in a pseudotyped rHSV particle is a Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped rHSV particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped rHSV particles. In other embodiments, the foreign viral capsid protein may be from a different HSV serotype. For example, an HSV-1 vector may contain one or more HSV-2 capsid proteins. Different HSV serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes include without limitation, central nervous system and neurons (*e.g.* HSV-1). For further description, see Manservigi, R. *et al.* (2010) *Open Virol J* 4:123-156, Kay, M. *et al.* (2001) *Nat. Med.* 7(1):33-40, and Meignier, B. *et al.* (1987) *J. Infect. Dis.* 155(5):921-930.

V. Production of Viral Vectors

[0129] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids. rAAV production cultures for the production of rAAV virus particles all require; 1) suitable host cells, including, for example, human-derived cell lines such as HeLa, A549, or 293 cells, or insect-derived cell lines such as SF-9, in the case of baculovirus production systems; 2) suitable helper virus function, provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus,

baculovirus, or a plasmid construct providing helper functions; 3) AAV rep and cap genes and gene products; 4) a transgene (such as a therapeutic transgene) flanked by at least one AAV ITR sequences ; and 5) suitable media and media components to support rAAV production. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (e.g., Spodoptera frugiperda (Sf9) cells).

[0130] Suitable rAAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, rAAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of rAAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of rAAV in production cultures.

[0131] In some aspects, the invention provides methods for preparing rAAV particles with reduced empty capsids comprising a) culturing host cells under conditions suitable for rAAV production, wherein the cells comprise i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR, ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and iii) nucleic acid encoding AAV helper virus functions; b) lysing the host cells to release rAAV particles; c) isolating the rAAV particles produced by the host cell; and d) analyzing the rAAV particles for the presence of empty capsids

and/or rAAV particles with variant genomes by analytical ultracentrifugation as described above. In some embodiments, the p5 promoter of the nucleic acid encoding AAV rep and cap regions is located 3' to the rep and/or cap coding region. In some embodiments, the nucleic acid encoding AAV rep and cap coding regions is plasmid pHLP, pHLP19, or pHLP09 (see US Patent Nos. 5,622,856; 6,001,650; 6,027,931; 6,365,403; 6,376,237; and 7,037,713; the content of each is incorporated herein in its entirety). In some embodiments, the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

[0132] rAAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, rAAV production cultures include attachment- dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. rAAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0133] rAAV vector particles of the invention may be harvested from rAAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of rAAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0134] Numerous methods are known in the art for production of adenoviral vector particles. For example, for a gutted adenoviral vector, the adenoviral vector genome and a helper adenovirus genome may be transfected into a packaging cell line (*e.g.,* a 293 cell line). In some embodiments, the helper adenovirus genome may contain recombination sites flanking its packaging signal, and both genomes may be transfected into a packaging cell line that expresses a recombinase (*e.g.,* the Cre/loxP system may be used), such that the adenoviral vector of interest is packaged more efficiently than the helper adenovirus (see, *e.g.,* Alba, R. *et al.* (2005) *Gene Ther.*

12 Suppl 1:S18-27). Adenoviral vectors may be harvested and purified using standard methods, such as those described herein.

[0135] Numerous methods are known in the art for production of lentiviral vector particles. For example, for a third-generation lentiviral vector, a vector containing the lentiviral genome of interest with gag and pol genes may be co-transfected into a packaging cell line (*e.g.,* a 293 cell line) along with a vector containing a rev gene. The lentiviral genome of interest also contains a chimeric LTR that promotes transcription in the absence of Tat (see Dull, T. *et al.* (1998) *J. Virol.* 72:8463-71). Lentiviral vectors may be harvested and purified using methods (*e.g.*, Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011) described herein.

[0136] Numerous methods are known in the art for production of HSV particles. HSV vectors may be harvested and purified using standard methods, such as those described herein. For example, for a replication-defective HSV vector, an HSV genome of interest that lacks all of the immediate early (IE) genes may be transfected into a complementing cell line that provides genes required for virus production, such as ICP4, ICP27, and ICP0 (see, *e.g.,* Samaniego, L.A. *et al.* (1998) *J. Virol.* 72:3307-20). HSV vectors may be harvested and purified using methods described (*e.g.*, Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79).

VI. Purification of rAAV Vectors

[0137] At harvest, rAAV production cultures of the present invention may contain one or more of the following: (1) host cell proteins; (2) host cell DNA; (3) plasmid DNA; (4) helper virus; (5) helper virus proteins; (6) helper virus DNA; and (7) media components including, for example, serum proteins, amino acids, transferrins and other low molecular weight proteins. In addition, rAAV production cultures further include rAAV particles having an AAV capsid serotype selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAV9, AAV10, AAVrh10, AAV11, or AAV12. In some embodiments, the rAAV production cultures further comprise empty AAV capsids (*e.g.,* a rAAV particle comprising capsid proteins but no rAAV genome). In some embodiments, the rAAV production cultures further comprise rAAV particles comprising variant rAAV genomes (*e.g.,* a rAAV particle comprising a rAAV genome that differs from an intact full-length rAAV genome). In

some embodiments, the rAAV production cultures further comprise rAAV particles comprising truncated rAAV genomes. In some embodiments, the rAAV production cultures further comprise rAAV particles comprising AAV-encapsidated DNA impurities.

[0138] In some embodiments, the rAAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0139] In some embodiments, the rAAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0140] rAAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the rAAV particles; rAAV capture by apatite chromatography; heat inactivation of helper virus; rAAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and rAAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify rAAV particles are found, for example, in Xiao *et al.*, (1998) *Journal of Virology* 72:2224-2232; US Patent Numbers 6,989,264 and 8,137,948 and WO 2010/148143. Methods to purify adenovirus particles are found, for example, in Bo, H *et al.*, (2014) *Eur. J. Pharm. Sci.* 67C:119-125. Methods to purify lentivirus particles are found, for example, in Segura MM, *et al.*, (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for example, in Goins, WF et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79.

EXAMPLES

[0141] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Characterization of recombinant adeno-associated viral vector preparations by analytical ultracentrifugation

[0142] Adeno-associated viruses (AAV) have features that make them attractive as vectors for gene therapy. Wild-type AAV consists of two open reading frames (rep and cap) which code for all structural and regulatory elements required for assembly, replication and infection. The rep ORF codes for Rep 78 and 68 proteins which have genome replication functions as well as Rep 52 and 40 proteins which are involved in single strand replication and packaging. The cap ORF codes for the three structural capsid proteins: VP1, VP2 and VP3. Recombinant AAV vector is typically produced by the triple transfection method using the "gutless" vector approach (Xiao, X, *et al.*, 1998, *J. Virol.* 3:2224-2232). The rep and cap genes are replaced with the therapeutic gene and its regulatory elements sandwiched between a 5' and 3' inverted terminal repeat (ITR), the rep and cap genes are provided in trans on a separate plasmid and a third plasmid contributes required adenoviral helper genes. It is postulated that the viral capsids are fully assembled and the ITR flanked vector genome is then inserted into the capsid via a capsid pore (Myers, MW & Carter, BJ, 1980, *Virology*, 102:71-82). The resulting population of capsids contains both non-genome containing capsids (empty capsids) as well as genome containing capsids. In addition, capsids may contain incomplete portions of the recombinant viral genome. The vector prep may then purified by affinity chromatography to isolate the capsids from the cellular debris and can be further processed to enrich for intact vector by anion exchange chromatography.

[0143] Based on their recent approval for use in gene therapy, adeno-associated viral (AAV) vectors have emerged as an important class of novel biopharmaceutical drug products. The generation of AAV vector products requires an analytical method that monitors product quality

with regard to homogeneity, purity, and consistency of manufacturing, yet to date no method to support AAV vector characterization has been established. To meet this demand, the potential use of analytical ultracentrifugation (AUC) as a technique to characterize the homogeneity of AAV vectors was investigated.

Methods

Sample preparation

[0144] In order to support accurate AUC assessment, vector product (AAV2-transgene 2) was highly purified, suitably buffered, and concentrated to greater than 5 x 10^{11} vg/mL. To achieve this, cell supernatants runs were purified using AVB affinity chromatography (GE Healthcare) and buffer-exchanged into PBS, pH 7.2 using a 10K MWCO Slide-a-Lyzer (Thermo Scientific). Product concentration was determined by optical density measurement at 260nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260nm from 0.1 to 1.0, either by direct dilution with PBS or further concentration using Amicon Ultra-0.5 /30K MWCO Centrifugal Filter Device.

Sedimentation velocity AUC data acquisition

[0145] Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis was performed using a ProteomeLab™ XL-I (Beckman Coulter). 400 µL sample was loaded into the sample sector of a two sector velocity cell, and 400 µL PBS was loaded into the corresponding reference sector. The sample was placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of 20°C and full vacuum were maintained for one hour. Sedimentation velocity centrifugation was performed at 20,000 RPM, 20°C, 0.003 cm radial step setting, with no delay and with no replicates. Absorbance (260 nm) and Raleigh interference optics were used to simultaneously record radial concentration as a function of time until the smallest sedimenting component cleared the optical window (1.2 hour). Assay throughput was limited to a single sample per run based on absorbance scan collection times of greater than one minute, as well as the large size and rapid sedimentation of AAV.

AUC data analysis

[0146] The percent full capsid was determined by analyzing approximately 75 scans from each detection method using the SEDFIT (NIH/see worldwide web at analyticalultracentrifugation.com) continuous size C(S) distribution model. Second (2nd) derivative regularization was applied to the fitting with a confidence level of F statistic=0.68. The following C(S) parameters were held constant: resolution=200S, S min=1, S max=200 and frictional ratio=1.0. RI and TI noise subtractions were applied, and the meniscus position was allowed to float, letting the software choose the optimal position. This model fit the data to the Lamm equation, and the resulting size distribution was a "distribution of sedimentation coefficients" that looked like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) were determined for each component in the distribution. Each AUC run was an independent assay, and each analysis was monitored for the following attributes to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\ nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans.

Absorbance optics (260 nm)

[0147] Extinction coefficients were used to calculate molar concentration and the actual percent value of the intact vector peak from absorbance data. Molar absorbance extinction coefficients for both empty capsids ($\epsilon_{260}/_{capsid}$=3.72e6) and intact vector ($\epsilon_{260}/_{vector}$=3.00e7) were calculated based on published formulae (Sommer *et al.* (2003) *Mol Ther.*, 7:122-8). Extinction coefficients were available for empty capsid and intact vector peaks. The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.,* 78:1606-19. Molar concentration of both intact vector and empty capsid were calculated using Beer's Law, and the percentage of full capsid was calculated from these values. Values were reported in terms of the percentage of full capsid.

Generation of an AUC standard curve

[0148] Because it is not possible to determine empirically the extinction coefficient of fragmented genomes of unknown size and sequence, a relationship between S value and genome

size was established. To achieve this, rAAV vector preps with encapsidated viral genomes of known nucleotide size were analyzed by AUC, and a corresponding S value was determined as described above.

Production of rAAV by transient transfection

[0149] Recombinant AAV vector was produced by the triple transfection method using the "gutless" vector approach (Xiao *et al.* (1998) *J. Virol.*, 3:2224-32). In this approach, the rep and cap genes were replaced with the therapeutic gene and its regulatory elements, both sandwiched between a 5' and 3' inverted terminal repeat (ITR). The rep and cap genes were provided in trans on a separate plasmid, and a third plasmid contributed the required adenoviral helper genes. Without wishing to be bound to theory, it is postulated that the viral capsids are fully assembled, and the ITR flanked vector genome is then inserted into the capsid via a capsid pore (Myers & Carter (1980) *Virology*, 102:71-82). The resulting population of capsids contained both non-genome-containing capsids (empty capsids) and genome-containing capsids.

Production of rAAV by producer cell platform

[0150] The AAV producer cell line is an alternative production platform used to generate clinical rAAV vectors. With this method, a HeLa S3 cell, adapted to growth in suspension, was engineered to have integrated copies of the AAV rep and cap genes required for vector replication and packaging, in addition to vector sequences and the selectable marker (*see, e.g.*, Puro: Thorne *et al.* (2009) *Hum. Gene Ther.*, 20:707-14). Once infected with WT Adenovirus, which provides the helper functions required for replication, the cell produced recombinant AAV vector as well as adenovirus, which was subsequently removed during the purification process using ion-exchange chromatography.

Other methods

[0151] Synthetic transgenes were cloned into a plasmid that contained a promoter of choice and bovine growth hormone polyadenylation signal sequence (polyA). The entire transgene expression cassette was then cloned into previral plasmid vector pAAVDC64 containing AAV2 inverted terminal repeats. The total size of the resulting AAV genomes in the respective expression plasmids (including the region flanked by ITRs) was 4 - 4.6 kb. The recombinant

vectors were produced by triple transfection of 293 cells using helper plasmids expressing rep2/cap sequences and Adenovirus helper functions, pAd Helper (Stratagene, La Jolla, CA USA) The rep/cap helper expressed rep from AAV serotype 2, while the cap sequence encoded one of the following sequences: AAV cap 1, 2, 5, 9, or rh8R. Vectors were purified by affinity chromatography and in some cases were further purified to remove empty particles (see, *e.g.,* Qu *et al.* (2007) J. *Virol. Methods.* 140:183-92).

Results

[0152] Analytical ultracentrifugation (AUC) using classical boundary sedimentation velocity was used to reveal the particle heterogeneities of recombinant adeno-associated virus (rAAV) vector preps. A mixture containing 20% rAAV2 particles with the full genome and 80% empty capsids was created by mixing together purified empty capsids and purified genome-containing capsids at defined ratios. The empty and full capsids were generated by $CsCl_2$ gradient purification of a mixture of empty and full capsids following triple transfection production. To monitor the movement of rAAV2 particles in response to a centrifugal force, this mixture of rAAV2 capsids was scanned at an absorbance of 260nm along a centrifugal field at defined time intervals. **FIG. 1A** shows a representative scanning profile following centrifugation of the AAV2 mixture at 20,000 rpm for 1.2 hrs (until the smallest sedimenting species cleared the optical window). Scans represented the acquisition of concentration data as a function of radius *r*, at times *t*, to yield a series of concentration scans that revealed the complete migration pattern of constituent vector particles in the rAAV2 vector prep. In these sigmoidal curves or boundaries, the leading edge of the curve represented the faster sedimenting species (*i.e.*, the genome-containing rAAV2 capsid), and the trailing edge of the curve represented the slower sedimenting species (*i.e.*, the "empty" rAAV2 capsids) (**FIG. 1A**).

[0153] Plotting the differential sedimentation coefficient distribution value, C(S), versus the sedimentation coefficient (in Svedberg units, S) yielded distinct peaks with unique sedimentation coefficients for both the empty and genome-containing capsid species (**FIG. 1B**). The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.,* 78:1606-19. In order to calculate molar concentrations and percent value for each capsid species from the absorbance data, extinction coefficients were used according to Table 2.

Table 2. Extinction coefficients and molar concentrations for capsid species

Species	Signal (Abs $_{260nm}$)	ε_{260nm}	Molar concentration (M)	Relative abundance (%)
Peak 1	0.0479	3.73E+06	1.28E-08	79
Peak 2	0.0909	2.59E+07	3.51E-09	21
Sum			1.63E-08	

[0154] Molar absorbance extinction coefficients for both the empty capsid (ε_{260} /$_{capsid}$ = 3.72e6) and genome containing capsid (ε_{260} /$_{capsid}$ = 3e7) were calculated using genome size and published formulae (Sommer *et al.*, 2003). Molar concentrations of both genome-containing and empty capsids were then calculated using Beer's Law. The molar concentration of each species was used to calculate its relative abundance, expressed as a percentage of total capsids (**FIG. 1**). These results demonstrated that AUC may be used to accurately distinguish and quantify empty capsids and genome-containing capsids from a heterogeneous vector preparation.

[0155] For the rAAV2 vector prep shown in **FIG. 1**, capsids containing a full genome were represented by the peak sedimenting at 94S and accounted for 21% of the vector prep. Empty capsids sedimented with an S value of 64S and accounted for 79% of the vector prep. These sedimentation coefficient values were confirmed by AUC analysis of pure populations of empty (**FIG. 2A**) or genome containing particles (**FIG. 2B**). The AUC profile of a pure population of rAAV2 empty capsids revealed a single peak with a sedimentation coefficient of 64S, whereas the AUC profile of a pure population of rAAV2AUC genome-containing capsids revealed a single peak with a higher sedimentation coefficient of 94S. These results agreed with the values generated from a heterogeneous preparation and further confirmed that AUC methods may be used to quantify genome-containing and empty AAV capsids from a heterogeneous preparation containing both species.

[0156] The AUC method was further assessed for reproducibility by performing five independent AUC runs of the same vector sample (scAAV2/9 LP2), as shown in Table 3. The sedimentation coefficients for both genome-containing and empty AAV2 capsids were highly reproducible, yielding coefficients of variation from 0.5-0.6%. Similarly, the relative abundance (expressed as a percentage of the total) of genome-containing capsids was determined with a coefficient of variation of approximately 2%. These results indicated that the AUC method for

quantifying genome-containing and empty AAV2 capsids yields highly reproducible and consistent values.

Table 3. Five independent assays on scAAV2/9 LP2 sample.

AUC Run	Empty Capsid, Peak 1 (S)	Full Capsid, Peak 3	
		% Full Capsids	S
20110927A	64.3	34.1	84.0
20111003A	64.6	34.2	84.6
20111005A	65.1	35.0	84.5
20111005B	64.2	35.2	83.8
20111011A	64.1	33.5	84.5
Mean	64.5	34.4	84.3
Standard Deviation	0.4	0.7	0.4
% CV	0.6	2.1	0.5

Example 2: Comparison of interference and absorbance detection methods for AUC

[0157] An alternative optical detection method for AUC, Rayleigh Interference Optics, was also evaluated. This detection method measures the sample concentration based on refractive index differences between a reference solution and the AAV containing sample. Like absorbance detection, interference detection can be applied to any rAAV regardless of the sequence of the genome. Unlike absorbance detection, which requires an extinction coefficient, interference detection yields integrated peaks that are directly proportional to concentration.

[0158] Pure populations of empty and genome-containing AAV2 capsids were mixed at a 1:1 ratio and analyzed by AUC using both interference (**FIG. 3A**) and absorbance detection methods (**FIG. 3B**). Interference detection revealed two populations of AAV capsids at the approximate expected ratios of 43% empty and 57% genome-containing (**FIG. 3A**). Both detection methods yielded similar abundance ratios. However, comparing the peak sizes generated by both methods illustrates the disconnect between peak height and concentration with absorbance detection (compare size of "empty capsid" peaks in **FIG. 3A** and **3B**). The data generated by both methods are compared in Table 4. The ratio of absorbance signal to interference signal (A_{260nm}/IF) can be used in a fashion analogous to the 260/280 ratio of absorbance data, and this assisted in identifying peaks in the C(S) distribution.

Table 4. S values and relative abundance generated by absorbance and interference detection.

Peak	Absorbance (260 nm)		Interference			A_{260nm}/IF
	Sedimentation coefficient (S)	Relative abundance (%)	Sedimentation coefficient (S)	Signal (fringes)	Relative abundance (%)	
Peak 1	63	47	62	0.080	43	0.41
Peak 2	93	53	92	0.104	57	2.38
Sum				0.184		

[0159] Although interference optics offers precision and resolution, it may require a high concentration of sample. Moreover, interference optics may be affected by a mismatch between the reference and AAV sample buffer. AAV samples, however, typically contain a low protein concentration, and it may be necessary to completely match the AAV sample and reference buffers.

Example 3: Influence of production method on AAV vector heterogeneity

[0160] The previous examples demonstrated that the AUC method is a highly accurate and reproducible way to resolve and quantify empty and genome-containing AAV capsids from a heterogeneous mixture. This capability could be advantageous for a variety of applications to evaluate the quality of AAV vector preparations. For example, a major problem in producing pure AAV vector preparations is the presence of capsids with partial or fragmented genomes. To illustrate the utility of the AUC method for resolving these species, AAV vectors generated by two different methods, termed the "triple transfection" and "producer cell line" methods, were analyzed by AUC.

[0161] An AAV2 vector harboring the transgene 2 was produced using either the triple transfection method (**FIG. 4**) or the producer cell line method (**FIG. 5**). For a description of these methods, see Example 1. Following chromatographic purification, both vector preps were analyzed by AUC. **FIG. 6A** shows a schematic of this AAV2 vector genome.

[0162] The AUC profiles of vector preps produced by these methods were remarkably different. Using the producer cell line method, 74% of capsids contained a full genome, represented by the 92S species (**FIG. 6B**). 19% were empty capsids, with the remainder containing a fragmented genome (75S species, 7%). In contrast, 82% of the capsids produced by

the triple transfection method were empty, 64S species (**FIG. 6C**), with 11% of capsids containing a fragmented genome (76S and 84S species) and only 8% of capsids having a full genome (94S).

[0163] These results demonstrate that vector preparations generated using the producer cell line technology may have high quality, containing predominantly capsids with a full genome. The vast majority of capsids produced by the triple transfection method are empty, with a greater proportion of capsids having a fragmented genome. These results also highlight the ability of the AUC method to resolve capsids with fragmented genomes, in addition to full genome-containing and empty capsids. Moreover, they illustrate the power of the AUC method in evaluating the quality and homogeneity of vector preparations.

Example 4: Use of AUC to assess removal of empty capsids from vector preps

[0164] The AUC method was evaluated as a tool to monitor removal of empty capsids using chromatographic methods (for methods, see Qu *et al.* (2007) *J. Virol. Methods,* 140:183-92). Separation of empty and genome-containing capsids was performed using anion exchange chromatography (**FIG. 7A**). AUC was performed on the resolved peaks to demonstrate that the genome-containing rAAV2 particles were enriched in the later fractions eluted from the resin ("Full Genome Capsid" in **FIG. 7A**).

[0165] As shown in **FIG. 7B**, AUC analysis revealed that genome-containing capsids represented 94% of the vector prep upon elution from the column. This later fraction yielded a single peak with a sedimentation coefficient of 92S. In contrast, the rAAV2 vector prep prior to the chromatographic step (**FIG. 7C**) had a substantial level of empty capsids. AUC analysis revealed two peaks with S values of 63 and 93, with the 63S peak (empty capsid) representing 52% of the total capsid population. These results show that chromatographic methods are highly effective in removing empty capsids from AAV vector preparations. Importantly, they demonstrate the utility of applying the AUC method to evaluate vector quality upon purification. The AUC method is a useful tool for evaluating different vector purification protocols or techniques.

Example 5: Assessment of viral genome integrity by the AUC method

[0166] As illustrated in Example 3, AAV vector preparations may contain capsids packaged with fragmented genomes, in addition to full genome and empty species. A major problem in generating homogeneous AAV preparations for therapeutic or research applications is the presence of capsids with fragmented genomes, which may result in aberrant or absent expression of transgenes of interest. Indeed, heterogeneity associated with AAV vector preps has been reported to result from packaging of fragmented genomes or AAV-encapsidated DNA impurities. (Kapranov *et al.* (2012) *Hum. Gene Ther.*, 23:46-55). Therefore, AUC was evaluated as a tool to quantify the aberrant packaging of fragmented genomes in rAAV vector preps.

[0167] Because it is not possible to determine empirically the extinction coefficient of fragmented genomes of unknown size and sequence, a relationship between S value and genome size was established. To achieve this, rAAV vector preps with encapsidated viral genomes of known size were analyzed by AUC, and their corresponding S values were determined, as shown in Table 5. A standard curve was then generated to correlate genome size and S value (**FIG. 8**). This demonstrated a highly linear relationship (R^2=0.9978) between sedimentation coefficient and genome size.

Table 5. S values for rAAV vectors with known genome size.

Predicted Trend Line		Calculated values	
Sedimentation coefficient (S)	**Genome size (# NT)**	**MW**	**Extinction coefficient (260 nm)**
(y)	**(x)**		
Empty capsid	N/A		3.72E+06
74	880	2.7E+05	9.17E+06
78	1421	4.4E+05	1.25E+07
82	1961	6.1E+05	1.59E+07
84	2232	6.9E+05	1.75E+07
88	2772	8.6E+05	2.09E+07
92	3313	1.0E+06	2.42E+07
96	3853	1.2E+06	2.76E+07
100	4394	1.4E+06	3.09E+07
104	4934	1.5E+06	3.43E+07
108	5475	1.7E+06	3.76E+07

[0168] To demonstrate the utility of AUC to detect genome fragments, a self-complementary vector comprising AAV2 ITRS, a minimal CBA promoter, and an EGFP transgene was packaged

into an AAV9 capsid (AAV2/9minCBAEGFP; see schematic in **FIG. 9A**). The vector particles were purified to eliminate empty capsids and analyzed by AUC. The standard curve was then used to assign genome size to each of the resolved genome containing capsids. Approximately 25% of the vector prep sedimented as a 101S species, representing an encapsidated genome of ~ 4.3kb (**FIG. 9A**). This 101S peak represented the double stranded dimeric vector genome, which has a predicted size of ~4.3kb. However, the majority of the vector prep (75%), sedimented with an S value of 82, which corresponds to a vector genome size of ~ 2kb (**FIG. 9A**), consistent with packaging of the single stranded monomer. The packaging of monomeric genomes with self-complementary vectors is well documented and is often a result of inadvertent terminal resolution at spurious "trs like" sequences despite the presence of an ITR with a mutated D sequence (McCarty *et al.* (2001) *Gene Ther.,* 8:1248-54).

[0169] **FIG. 9B** shows an alkaline Southern blot of the same vector, scAAV9 EGFP, which revealed two vector populations with genomes ~ 4.3kb and ~2kb in size, corroborating the AUC data in **FIG. 9A**. The Southern blot also confirmed that the monomeric viral genome was preferentially packaged over the dimeric genome. Interestingly, AUC analysis of a single stranded AAV9 EGFP vector (~4kb) revealed a single predominant peak with a measured S value of 99S, corresponding to approximately 4.1kb by the standard curve and 84% of capsid abundance (**FIG. 9C**). These results suggest that single stranded AAV vectors may be packaged in a more homogeneous manner than double stranded vectors. Again, in agreement with the AUC method, Southern blot analysis of this vector prep revealed homogeneous encapsidation of a viral genome of the predicted size of ~4kb (lane 2, **FIG. 9B**). These results demonstrate that the AUC method may be used to measure the size of AAV vector genomes, yielding genome size data in agreement with the standard Southern blotting technique. Using the AUC method, single stranded AAV vectors were found to produce more homogeneous vector preparations than double stranded ones. These results show that the AUC method is a powerful tool to identify and quantify capsid species with incomplete genomes from vector preparations.

Example 6: Use of AUC to assess factors that influence packaging of vector genomes

[0170] The AUC method was next used as a tool to identify factors that influence the packaging of intact AAV vector genomes.

[0171] As discussed in Example 3, the production of rAAV vectors by transient transfection methods requires the use of three plasmids including a rep/cap helper, an ITR vector plasmid, and a pAd helper (see **FIG. 4**). AUC was used to assess the effect of the rep/cap helper on vector genome packaging for both single stranded and self-complementary AAV vectors. First, a self-complementary AAV vector harboring an EGFP transgene (**FIG. 10A**) was produced using one of two methods. In the first method (**FIG. 10B**), a helper plasmid was used in which rep 78/68 expression was driven by the endogenous p5 promoter ("WT Rep" construct). In the second method (**FIG. 10C**), the helper was modified such that 78/68 expression was reduced by moving the p5 promoter downstream of the cap2 sequence as well as mutating the TATA box ("pHLP Rep" construct). The full scAAV2 EFGP capsid was predicted to have a sedimentation coefficient of 100S in dimeric genome form and 80S in monomeric genome form (**FIG. 10A**).

[0172] AUC analysis of these scAAV2EGFP vector preps revealed a significant difference in vector genome packaging. In the presence of reduced rep78/68 (pHLP), more than half (55%) of the vector prep contained dimeric genomes, represented by the 100S species (**FIG. 10C**). This was the expected sedimentation coefficient for a capsid containing a dimeric genome of 4.4kb. In contrast, the scAAV2EGFP prep generated with the full complement of rep78/68 had significantly less packaged dimeric genomes (26%), with the majority of the capsids containing monomeric genomes and sedimenting at 80S (**FIG. 10B**). These results uncovered a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid, leading to reduced rep 78/68 protein levels.

[0173] A single stranded AAV5 Factor IX vector, AAV5FIX, (**FIG. 11A-B**) and a single stranded AAV5hSMN vector (**FIG. 11C-D**) were generated using rep/cap helpers that differed in rep expression as described above, but cap sequences of AAV2 were replaced by cap sequences of AAV5. Based on the nucleotide size of the FIX expression cassette (4.3kb), the predicted sedimentation coefficient for the AAV5 FIX vector capsid was approximately 101S. AUC analysis of AAV2/5FIX made in the presence of reduced rep78/68 ("pHLP19 Rep") revealed a homogenous profile with the majority of the vector (90%) sedimenting with an S value of the expected size, ~101S (**FIG. 11A**). In contrast, AAV5 FIX vector generated using a rep/cap5 helper expressing wild-type levels of rep 78/68 proteins ("WT Rep") generated a strikingly different AUC profile (**FIG. 11B**). Instead of a predominant peak at 101S, this profile revealed more capsid heterogeneity, with the majority of the AAV5 FIX (80%) sedimenting at a lower S

value of 86S, likely representing packaging of a fragmented genome. Moreover, in this vector sample only 15% of the AAV5 FIX vector capsids sedimented at the correct S value of ~ 104S (**FIG. 11B**).

[0174] AAV5SMN vectors made using these same wild-type and mutated p5 rep/cap helpers also had strikingly different AUC profiles. As seen with the single stranded AAV5FIX vectors, AAV5 SMN vectors generated in the presence of reduced rep78/68 showed less heterogeneity by AUC analysis, with a single capsid species sedimenting at the S value of 101S, consistent with packaging of a genome of the predicted size of ~ 4.4kb (**FIG. 11C**). In contrast, the AUC profile for the same vector genome packaged using "wild-type" levels of rep78/68 protein revealed three distinct AAV vector species, with sedimentation coefficients of 100S (predicted S value for the full vector genome of 4400nt), 92S (representing a fragmented genome of approximately 3300nt) and 80S (representing a fragmented genome of 2000 nucleotides) (**FIG. 11D**). These results confirm a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid using two additional AAV vectors.

[0175] Further analysis of the AAV5SMN and AAV5FIX vector preps was performed by Southern blot analysis of vector DNA. In agreement with the AUC method, Southern blot analysis of AAV5SMN generated with wild-type rep78/68 protein levels revealed packaging of full length (4.4kb) and fragmented (less than 4.4 kb) SMN genomes (**FIG. 12A**, lane 1). In contrast, the AAV5SMN vector generated in the presence of reduced rep78/68 protein contained largely capsids with a full length SMN genome (**FIG. 12A**, lane 2). Interestingly, a comparison of the two AAV5FIX vector preps by Southern analysis revealed the presence of a FIX full length genome even when vector was produced in the presence of wild-type levels of rep 78/68 (**FIG. 12B**, lane 2). However, AUC analysis of this AAV5FIX vector (**FIG. 11B**) showed that 80% of the capsids contained a fragmented genome (~ 3000 nucleotides), which were undetected by the FIX probe.

[0176] Further analysis of the FIX vector preps generated under the two experimental conditions was performed by generating probes to discrete regions of the vector plasmid, including regions of the backbone. A map of this vector is provided in **FIG. 13**. **FIG. 14** shows Southern blotting analyses using these probes to compare these FIX vector preps generated under different conditions. As shown in **FIG. 14A**, both vector preparations (pHLP rep, lane 1; WT rep,

lane 2) contained the hFIX transgene. However, **FIG. 14B** lane 2confirms that the vector genome species sedimenting at 86S observed in the WT Rep preparation (**FIG. 11B**) was a ~ 3kb fragment. Moreover, this species reacted with an Amp^R specific probe (**FIG. 14B**, lane 2), suggesting that packaging upstream of the 5'ITR had occurred in a rep dependent manner. In contrast, there was no evidence of an Amp^R containing fragment in the rAAV5 FIX vector preps that were generated in the presence of reduced levels of rep 68 / 78 (**FIG. 14B**, lane 1).

[0177] DNA impurities in the AAV FIX preps were also assessed by Q-PCR using primers and probes specific for Amp^R. By Q-PCR, approximately 35% Amp^R titer was detected in AAV5FIX vector preps generated in the presence of "wt" rep, in contrast to less than 1% when the same vector plasmid was used to generate AAV5FIX vector in the presence of reduced rep68/78 (data not shown). These results underscore the utility of AUC analysis for revealing the presence of packaged genomes that would otherwise go undetected by gene specific Southern blot analysis.

[0178] The packaging capacity of AAV vectors has been studied extensively, and although numerous reports have demonstrated successful transduction with vectors packaging oversized AAV genomes, the latter have been shown to be fragmented into subgenomic – length DNA. To further explore the applicability of the AUC method, the heterogeneity of AAV vectors produced using oversized genomes was evaluated. An expression cassette harboring the full length CBA promoter driving expression of the β-phosphodiesterase transgene was packaged as an oversized genome of 5.4kb (**FIG. 15A**) or as a wild type size genome of 4.6kb (**FIG. 15B**). To generate the 4.6kb genome, the CBA promoter was truncated by reducing the size of the intron as previously reported (Gray, SJ *et al.*, (2011) *Hum. Gene Ther.* 22(9):1143-1153).

[0179] As shown in **FIG. 15A**, the AUC profile of the AAV vector prep generated using the oversized vector genome demonstrated that nearly half of the vector prep sedimented as a 93S species, consistent with packaging a fragmented vector genome of approximately 3.5kb. 30% of the preparation was represented by another sub-genomic vector species of approximately 4.9kb sedimenting at 105S. There was no evidence of packaging of a full-length 5.4kb genome, which was predicted to sediment at 108-109S. In contrast, AUC analysis revealed that the same transgene under control of the abbreviated CBA promoter sedimented predominantly as a vector species of 102S, consistent with packaging of the predicted, full-length vector genome of 4.6 kb (**FIG. 15B**). These results demonstrate the utility of AUC analysis in profiling AAV vectors with

oversized genomes, and this profiling is critical, given the observed incidence of genome fragmentation.

[0180] This example demonstrated that the AUC method is highly effective in analyzing the genome size of AAV vector capsids in a heterogeneous preparation. By resolving genome-containing capsids by size (*e.g.,* dimeric and monomeric genomes, or partial fragments thereof), the AUC method represents a powerful tool for assaying the quality of AAV vector preps produced under different conditions. Moreover, the results from three distinct vector systems demonstrated that the AUC method is widely useful for quality control and optimization of conditions to yield improved AAV vector preparations. Importantly, the AUC method is able to detect fragmented genomes that are not detectable by Southern blot analysis. Whereas Southern blotting relies on the presence of DNA probe sequence for detection, the AUC method is sequence-independent. The AUC method has also been demonstrated to be an effective tool in analyzing oversized AAV genomes. In total, these results demonstrate the highly advantageous and effective implementation of AUC methods to analyze multiple types of AAV vector preparations, which have been found to display dramatically variable effects on genome packaging.

Example 7: Characterization of recombinant adenoviral vector preparations by analytical ultracentrifugation

[0181] Adenovirus (Ad) vectors have features that make them attractive as vectors for gene therapy. The generation of Ad vector products requires an analytical method that monitors product quality with regard to homogeneity, purity, and consistency of manufacturing. To meet this demand, the potential use of analytical ultracentrifugation (AUC) as a technique to characterize the homogeneity of Ad vectors was investigated.

Methods

Sample preparation

[0182] In order to support accurate AUC assessment, a recombinant adenovirus serotype 2 vector (Ad2) was prepared and highly purified ty CsCl gradient ultrafiltration to enrich for genome containing particles. Product concentration was determined by optical density

measurement at 260nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260nm from 0.1 to 1.0, either by direct dilution with PBS or further concentration using Amicon Ultra-0.5 /30K MWCO Centrifugal Filter Device.

Sedimentation velocity AUC data acquisition

Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis was performed using a ProteomeLab™ XL-I (Beckman Coulter). 400 µL sample was loaded into the sample sector of a two sector velocity cell, and 400 µL PBS was loaded into the corresponding reference sector. The sample was placed in the four-hole rotor and allowed to equilibrate in the instrument until a temperature of 20°C and full vacuum were maintained for one hour. Sedimentation velocity centrifugation was performed at 6,000 RPM, 20°C, 0.003 cm radial step setting, with no delay and with no replicates. Raleigh interference optics were used to simultaneously record radial concentration as a function of time until the smallest sedimenting component cleared the optical window (1.2 hour). Assay throughput was limited to a single sample per run based on absorbance scan collection times of greater than one minute, as well as the large size and rapid sedimentation of Ad2.

AUC data analysis

[0183] The percent full capsid was determined by analyzing approximately 75 scans from interference detection method using the SEDFIT (NIH/see worldwide web at analyticalultracentrifugation.com) continuous size C(S) distribution model. Second (2nd) derivative regularization was applied to the fitting with a confidence level of F statistic/ratio=0.68. The following C(S) parameters were held constant: resolution=250S, S min=10, S max=1500 and frictional ratio=1.86935. RI and TI noise subtractions were applied, and the meniscus position was allowed to float, letting the software choose the optimal position. This model fit the data to the Lamm equation, and the resulting size distribution was a "distribution of sedimentation coefficients" that looked like a chromatogram with the area under each peak proportional to concentration in units of Fringes or OD_{260} units. The sedimentation coefficient (in Svedberg units) and the relative concentration (in OD units) were determined for each component in the distribution. Each AUC run was an independent assay, and each analysis was monitored for the

following attributes to ensure quality of results: goodness of fit (rmsd), the ratio of $OD_{260\ nm}$/ interference signal in fringes (A260/IF ratio) for each peak, consistency of sedimentation coefficients for each species between runs, and overall quality of the scans. The rmsd of this representative example was 0.006584.

Results

[0184] Analytical ultracentrifugation (AUC) using classical boundary sedimentation velocity was used to reveal the particle heterogeneities of recombinant adenovirus serotype 2 vectdor (rAd2) vector preps. To monitor the movement of rAd2 particles in response to a centrifugal force, this mixture of rAd2 capsids was scanned using interference optics along a centrifugal field at defined time intervals. Scans represented the acquisition of concentration data as a function of radius *r*, at times *t*, to yield a series of concentration scans that revealed the complete migration pattern of constituent vector particles in the rAd2 vector prep. Plotting the differential sedimentation coefficient distribution value, C(S), versus the sedimentation coefficient (in Svedberg units, S) yielded distinct peaks with unique sedimentation coefficients rAd2 species (**FIG. 16**). The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys. J.,* 78:1606-19.

[0185] For the rAd2 vector prep shown in **FIG. 16**, 87.8% of the rAd2 vector preparation sedimented with an S value of 731, consistent with a vector preparation consisting predominantly of genome containing capsids. These data confirm that adenoviral particles can be resolved by AUC.

CLAIMS

What is claimed is:

1. A method of characterizing a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

2. A method to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and

c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes.

3. The method of claim 2 further comprising integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

4. A method to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

5. A method of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles, and

d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

6. A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

7. A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

8. A method of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles,

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d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

9. A method of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method of any one of claims 5-8, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

10. The method of any one of claims 4-9, wherein the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles.

11. The method of any one of claims 4-9, wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes.

12. The method of claim 11, wherein the capsid particles comprising variant sized genomes comprises truncated genomes, aggregates, recombinants and/or DNA impurities.

13. A method of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the

peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation.

14. The method of claim 13, wherein the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes.

15. The method of claim 14, wherein the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities.

16. The method of any one of claims 13-15 further comprising integrating the area under each peak in the C(S) distribution to determine the relative concentration of each species of recombinant viral particles.

17. A method of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the method of claim 16, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

18. The method of any one of claims 1-17, wherein the sedimentation of recombinant viral particles is monitored by absorbance.

19. The method of claim 18, wherein the absorbance is at about 230 nm, 260 nm or 280 nm.

20. The method of claim 18 or 19, wherein the absorbance is at about 260 nm.

21. The method of any one of claims 1-20, wherein the sedimentation of recombinant viral particles is monitored by interference.

22. The method of claim 21, wherein the interference is Rayleigh interference.

23. The method of any one of claims 1-22, wherein the preparation is an aqueous solution.

24. The method of claim 23, wherein the aqueous solution comprises a pharmaceutical formulation.

25. The method of claim 23 or 24, wherein the aqueous solution comprises a buffer.

26. The method of claim 25, wherein the buffer is at physiological pH.

27. The method of claim 25 or 26, wherein the buffer is at physiological osmolality.

28. The method of any one of claims 24-27, wherein the pharmaceutical formulation comprises phosphate buffered saline (PBS).

29. The method of claim 28, wherein the PBS has pH of about 7.2 and an osmolality of about 300 mOsm/L.

30. The method of any one of claims 24-29, wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

31. The method of any one of claims 1-30, wherein the C(S) values are determined by an algorithm that comprises Lamm equation solutions.

32. The method of claim 31, wherein the algorithm is the SEDFIT algorithm.

33. The method of any one of claims 1-32, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge.

34. The method of any one of claims 1-32, wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell.

35. The method of any one of claims 1-33, wherein sedimentation is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell.

36. The method of claim 34, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears an optical window.

37. The method of any one of claims 33-36, wherein at least 30 scans are used to monitor sedimentation of recombinant viral particles.

38. The method of claim 37, wherein about 30 scans are used to monitor sedimentation of recombinant viral particles.

39. The method of claim 37, wherein about 30 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

40. The method of claim 37, wherein about 30 to about 50 scans are used to monitor sedimentation of recombinant viral particles.

41. The method of claim 37, wherein about 50 to about 75 scans are used to monitor sedimentation of recombinant viral particles.

42. The method of claim any one of claims 31-41, wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68.

43. The method of claim 42, wherein the regularization is a second derivative regularization.

44. The method of claim 42, wherein the regularization is Max entropy regularization.

45. The method of any one of claims 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.90.

46. The method of any one of claims 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68 to about 0.99.

47. The method of any one of claim 42-44, wherein the regularization is applied to a fitting level with a confidence level of F statistic of about 0.68.

48. The method of any one of claims 31-47, wherein the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software.

49. The method of claim 48, wherein resolution is about 200S to about 1000S.

50. The method of claim 48 or 49, wherein resolution is about 200S.

51. The method of any one of claims 48-50, wherein S min is about 1.

52. The method of any one of claims 48-51, wherein Smax is about 100S to about 1000S.

53. The method of any one of claims 48-51, wherein Smax is about 200S to about 5000S.

54. The method of any one of claims 48-51, wherein Smax is about 200S.

55. The method of any one of claims 48-54, wherein the frictional ratio is left to float to a value determined by centrifugation software.

56. The method of claim any one of 48-54, wherein the frictional ratio is about 1.0.

57. The method of any one of claims 31-56, wherein radial invariant (RI) and time invariant (TI) noise subtractions are applied.

58. The method of any one of claims 1-57, wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds.

59. The method of claim 58, wherein the sedimentation of recombinant viral particles is monitored about every 10 seconds.

60. The method of claim 58, wherein the sedimentation of recombinant viral particles is monitored about every 60 seconds

61. The method of any one of claims 1-60, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm.

62. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 10,000 rpm.

63. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 10,000 rpm to about 20,000 rpm.

64. The method of claim 61, wherein the boundary sedimentation velocity is performed at about 15,000 rpm to about 20,000 rpm.

65. The method of any one of claims 1-64, wherein the boundary sedimentation velocity is performed at about 4°C to about 20 °C.

66. The method of claim 65, wherein the boundary sedimentation velocity is performed at about 4°C.

67. The method of any one of claims 1-66, wherein the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle.

68. A method of evaluating a process for the production of recombinant viral particles comprising the method of any one of claims 1 to 67, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles.

69. The method of claim 68, wherein the viral particles are rAAV particles.

70. The method of claim 69, wherein the rAAV particles are produced from a producer cell line.

71. The method of claim 69, wherein the rAAV particles are produced by triple transfection of i) nucleic acid encoding AAV rep and cap, ii) rAAV vector sequences, and iii) nucleic acid encoding adenovirus helper functions.

72. The method of claim 69, wherein the recombinant viral particles are produced by an AAV/HSV hybrid

73. The method of claim 69, wherein the recombinant viral particles are produced from a insect cell.

74. The method of claim 69, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding AAV vector sequences, AAV rep and cap coding regions, and AAV helper virus functions to a suitable host cell.

75. The method of claim 69, wherein the recombinant viral particles are produced by introduction of one or more nucleic acids encoding AAV vector sequences, AAV rep and cap

coding regions, and AAV helper virus functions to a suitable host cell, wherein the one or more nucleic acids are introduced to the cell using a recombinant helper virus.

76. The method of claim 75, wherein the recombinant helper virus is an adenovirus, a herpes simplex virus or a baculovirus.

77. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding adenovirus vector sequences and adenovirus replication and packaging sequences to a suitable host cell.

78. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding lentivirus vector sequences and/or lentivirus replication and packaging sequences to a suitable host cell.

79. The method of claim 68, wherein the recombinant viral particles are produced by transient transfection of nucleic acid encoding HSV vector sequences and/or HSV replication and packaging sequences to a suitable host cell.

80. A method for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising

a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant viral particles;

c) isolating the recombinant viral particles produced by the host cell; and

d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the methods of any one of claims 1 to 79.

81. The method of claim 80, wherein the p5 promoter is located 3' to the rep and/or cap coding region.

82. The method of claim 80 or 81, wherein the AAV helper virus functions comprise adenovirus E1A function, adenovirus E1B function, adenovirus E2A function, adenovirus VA function and adenovirus E4 orf6 function.

83. The method of any one of the preceding claims wherein the recombinant viral particles have been purified using one or more purification steps.

84. The method of claim 67, 69-76, 80-83 wherein the recombinant viral particles comprise a self-complementary AAV (scAAV) genome.

85. The method of claim 84, wherein the method is used to detect the presence of recombinant viral particles comprising the monomeric form of a scAAV genome or the dimeric form of a scAAV genome.

86. The method of any one of claims 67, 69-76, 80-85,wherein the recombinant viral particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1).

87. The method of any one of claims 67, 69-76, 80-86, wherein the recombinant viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, an AAV12 ITR, an AAV DJ ITR, a goat AAV ITR, a bovine AAV ITR, or a mouse AAV ITR.

88. The method of claim 86 or 87, wherein the AAV capsid comprises a tyrosine mutation or a heparin binding mutation,

89. The method of claim 67 or 68, where the recombinant viral particle is a recombinant adenoviral particle.

90. The method of claim 89, wherein the recombinant adenoviral particle comprises an capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, or porcine Ad type 3.

91. The method of claim 90, wherein the recombinant adenoviral particle comprises a variant of an adenovirus serotype 2 capsid or a variant of an adenoviral serotype 5 capsid.

92. The method of claim 67 or 68, where the recombinant viral particle is a recombinant lentiviral particle.

93. The method of claim 92, wherein the recombinant lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114 or variants therein.

94. The method of claim 67 or 68, where the recombinant viral particle is a rHSV particle.

95. The method of claim 94, wherein the HSV particle is an HSV-1 particle or an HSV-2 particle.

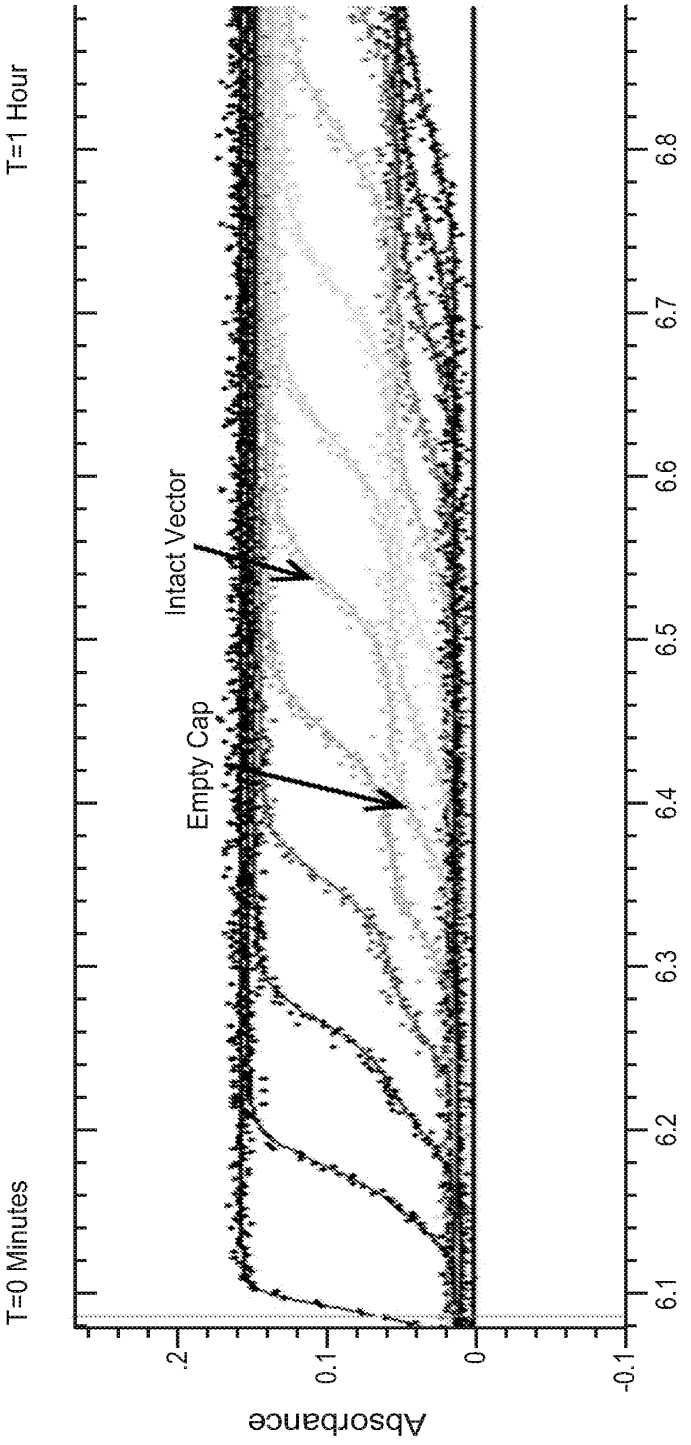

T=1 Hour
T=0 Minutes
Intact Vector
Empty Cap
Absorbance
.2
0.1
0
-0.1
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
Radius, cm

FIG. 1A

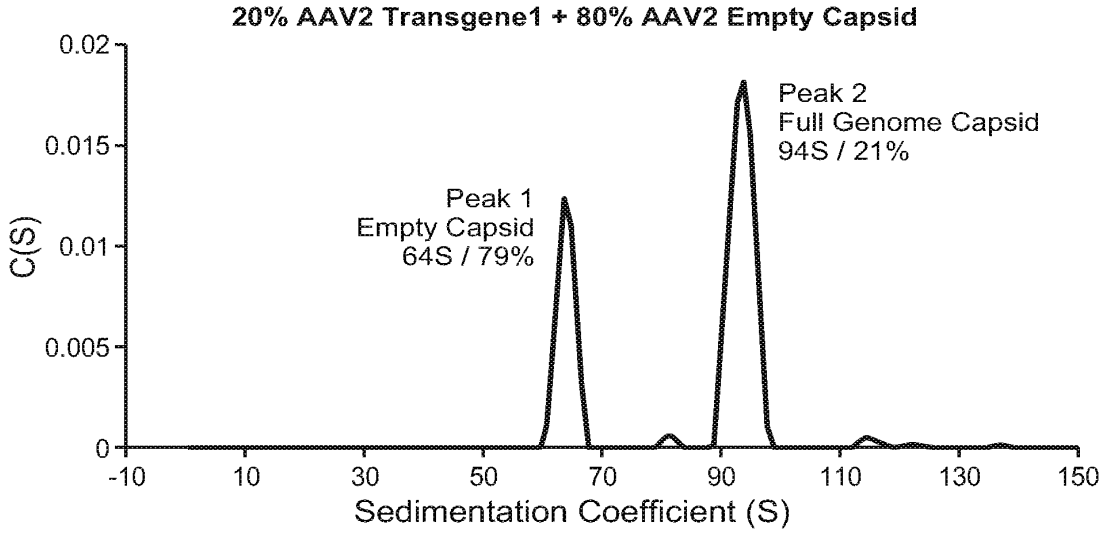

20% AAV2 Transgene1 + 80% AAV2 Empty Capsid
0.02
0.015
0.01
0.005
0
C(S)
Peak 1
Empty Capsid
64S / 79%
Peak 2
Full Genome Capsid
94S / 21%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 1B

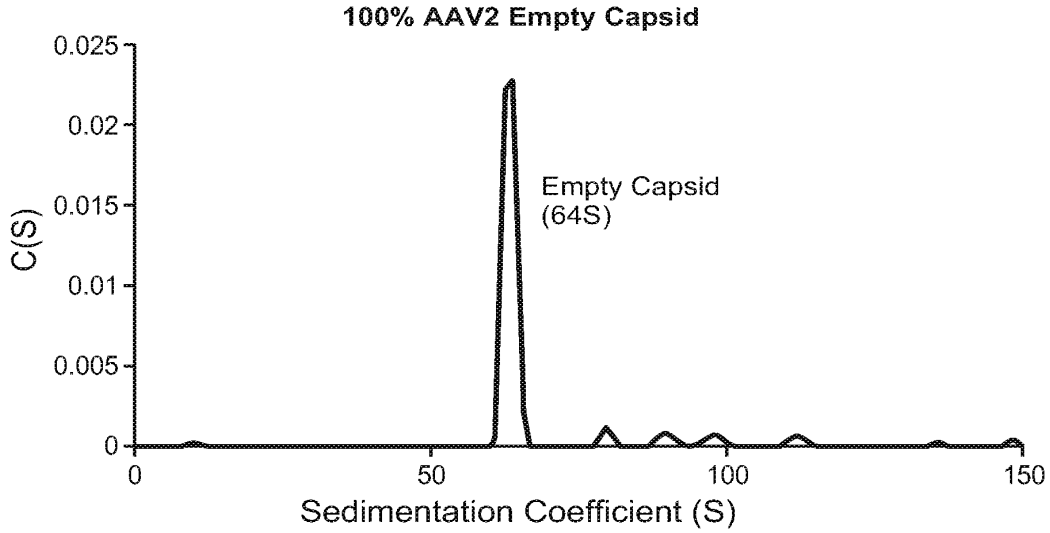

100% AAV2 Empty Capsid
0.025
0.02
0.015
0.01
0.005
0
C(S)
Empty Capsid
(64S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2A

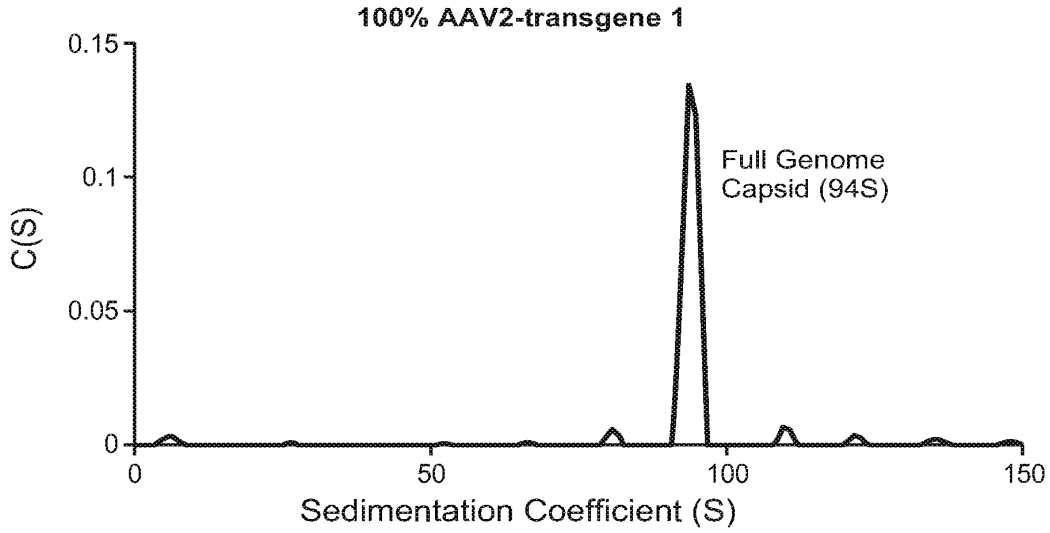

100% AAV2-transgene 1
0.15
0.1
0.05
0
C(S)
Full Genome
Capsid (94S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2B

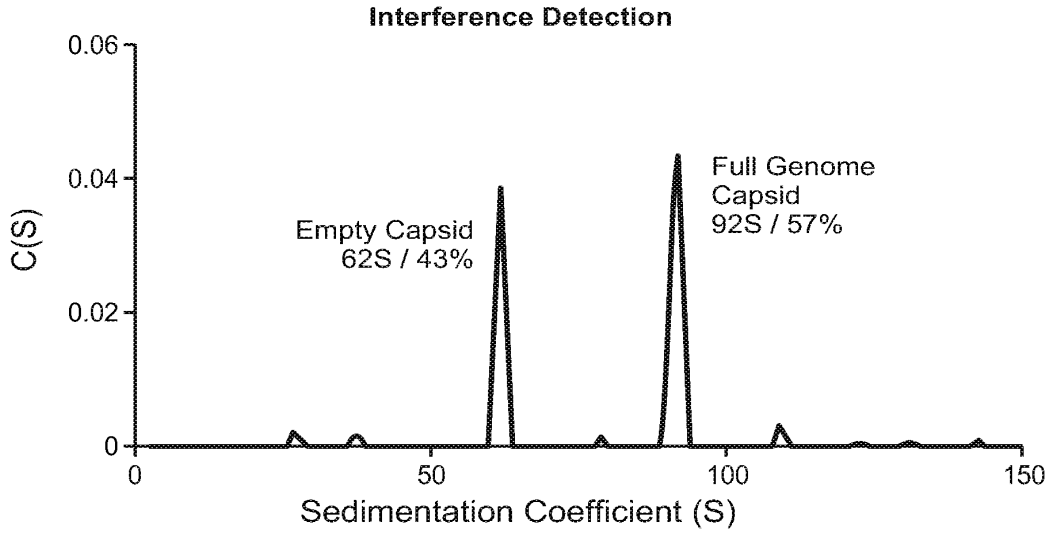

Interference Detection
0.06
0.04
0.02
0
C(S)
Empty Capsid
62S / 43%
Full Genome
Capsid
92S / 57%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3A

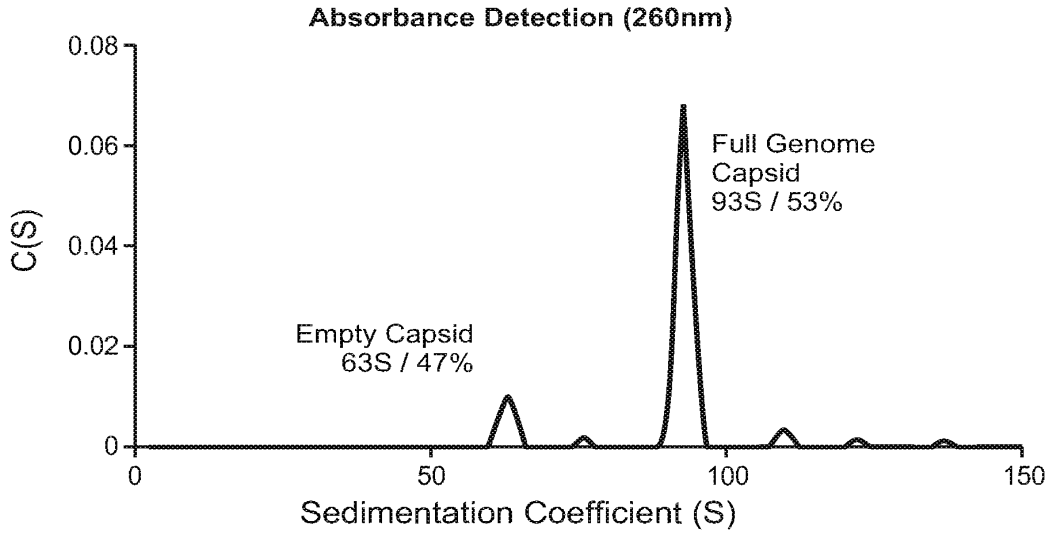

Absorbance Detection (260nm)
0.08
0.06
0.04
0.02
0
C(S)
Empty Capsid
63S / 47%
Full Genome
Capsid
93S / 53%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3B

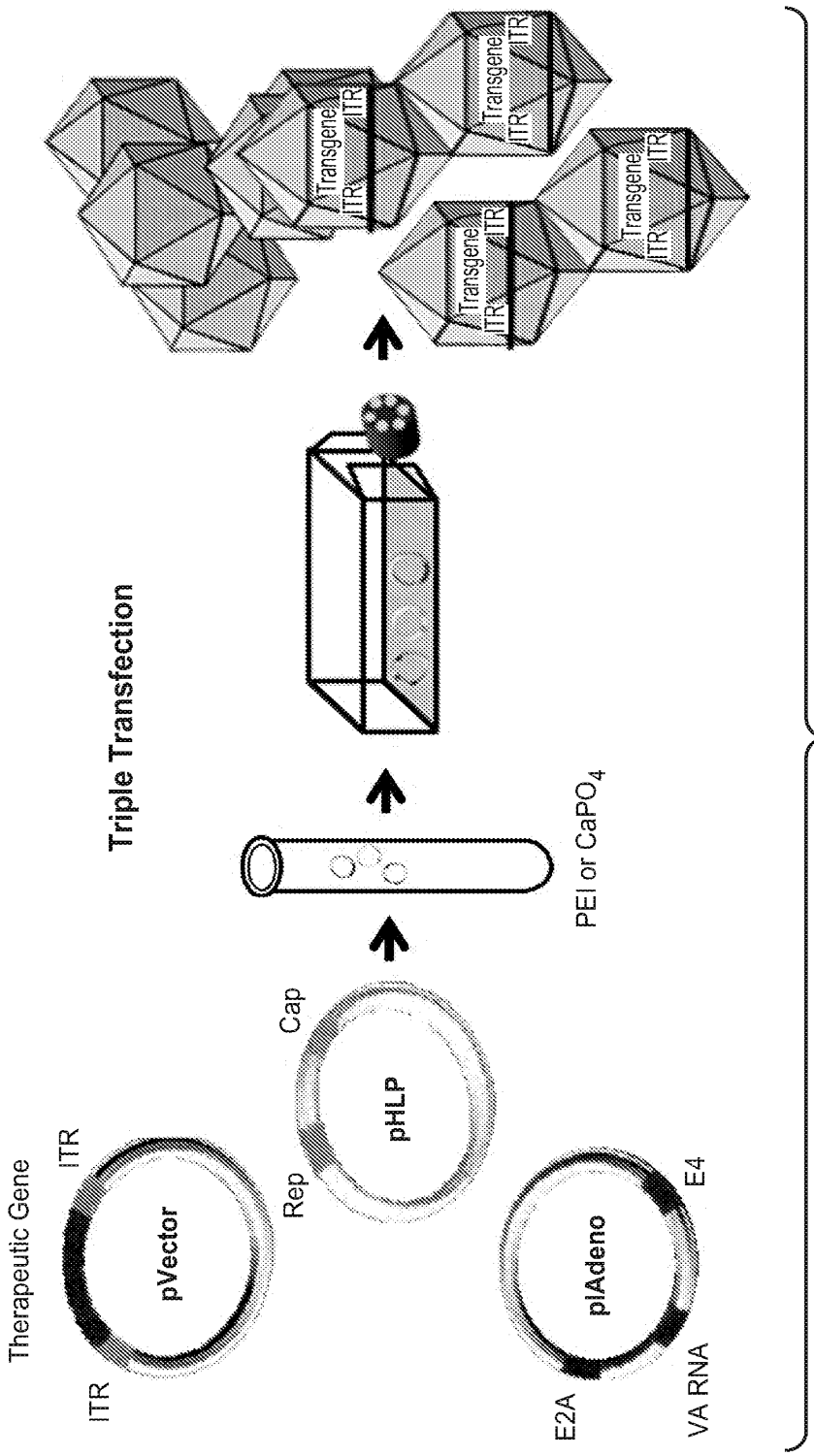

Transgene
ITR
Triple Transfection
PEI or CaPO4
Cap
pHLP
Rep
Therapeutic Gene
ITR
pVector
ITR
E4
pIAdeno
E2A
VA RNA

FIG. 4

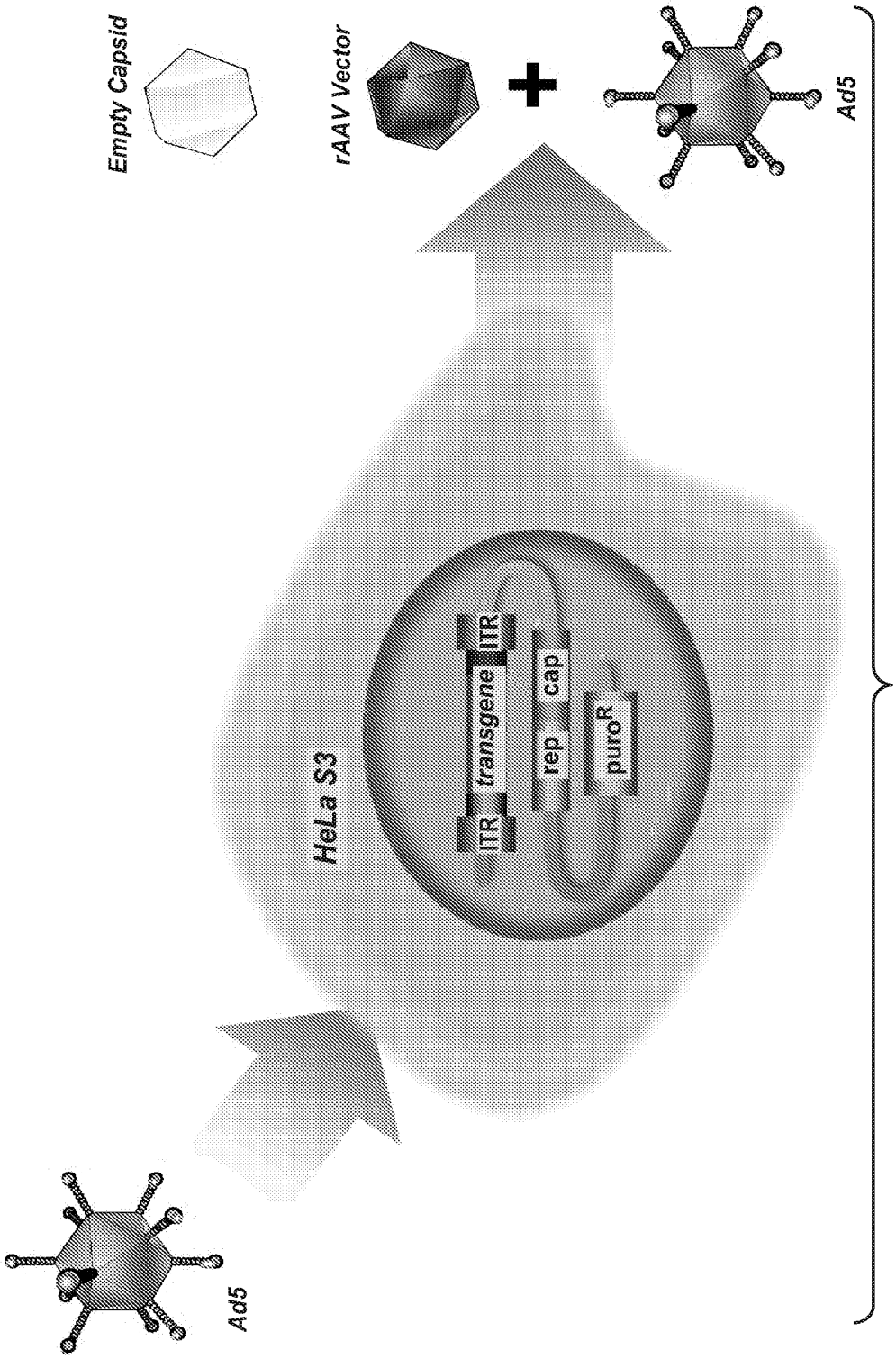
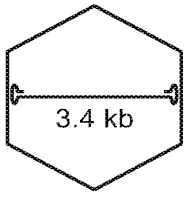

Empty Capsid
rAAV Vector
Ad5
HeLa S3
ITR
transgene
ITR
rep
cap
puroR
Ad5

FIG. 5

FIG. 6A



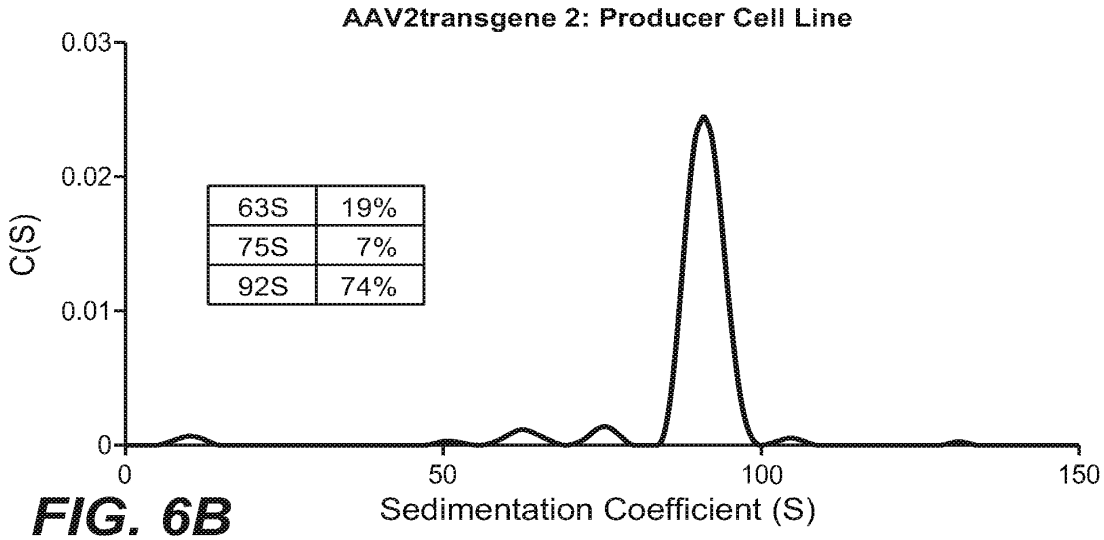


FIG. 6B

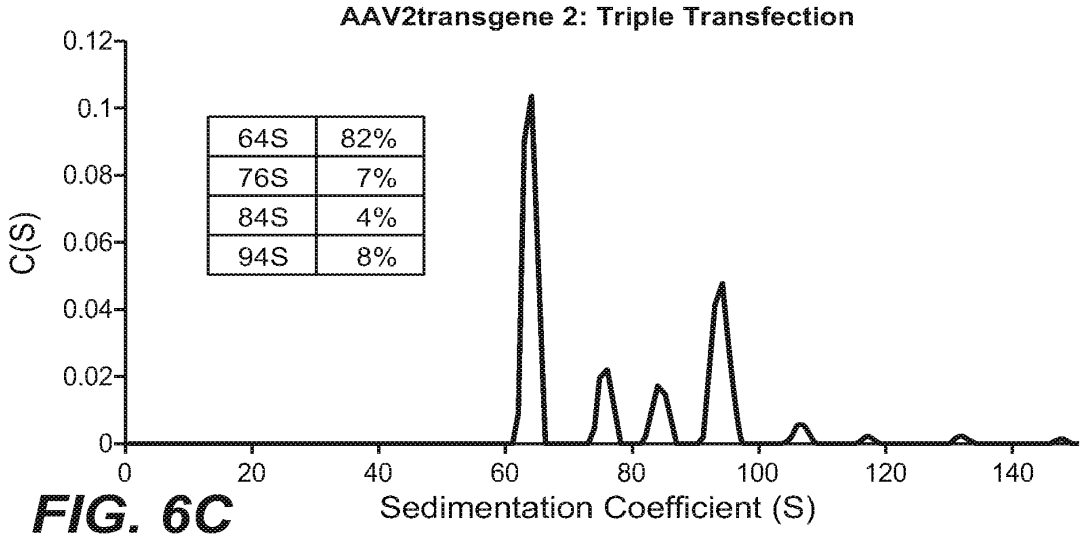


FIG. 6C

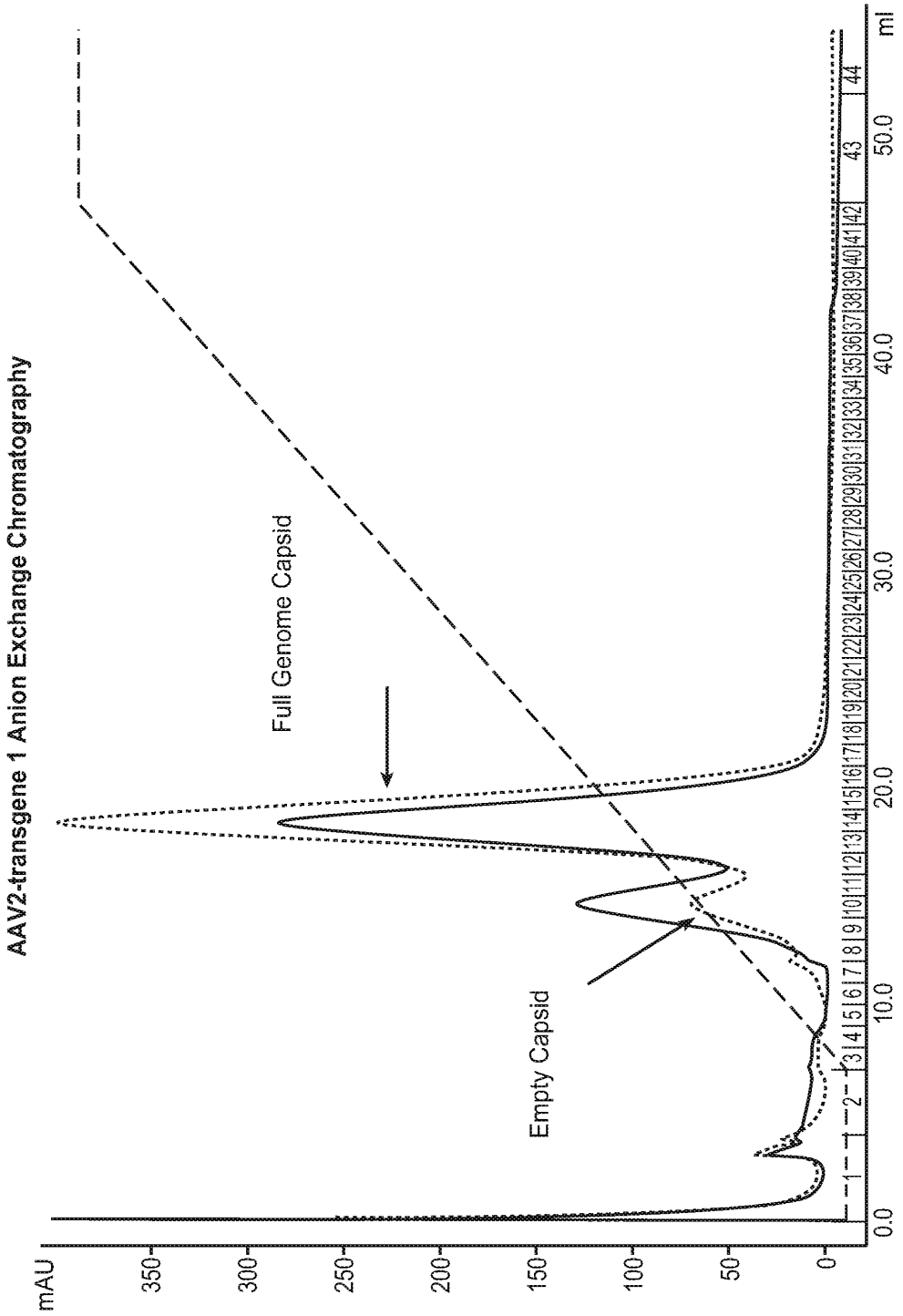

AAV2-transgene 1 Anion Exchange Chromatography
mAU
350
300
250
200
150
100
50
0
0.0
10.0
20.0
30.0
40.0
50.0
ml
Full Genome Capsid
Empty Capsid

FIG. 7A

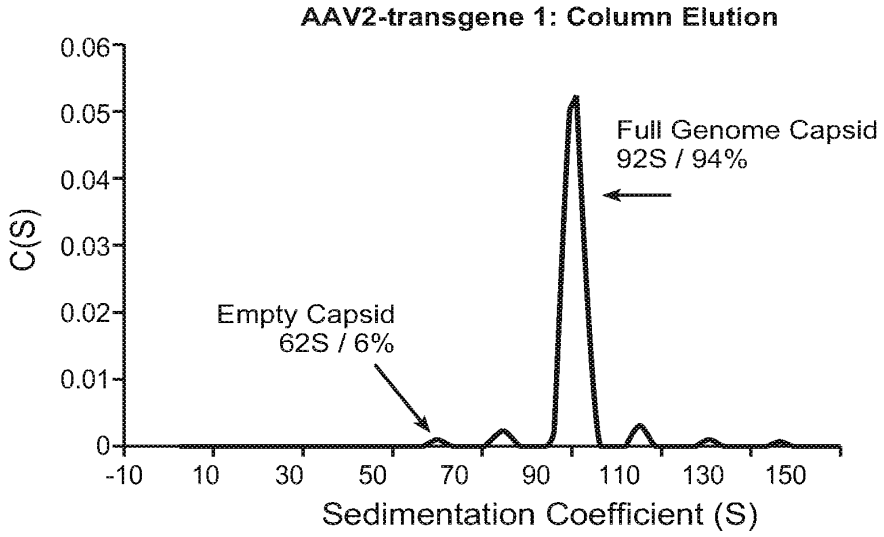

AAV2-transgene 1: Column Elution
0.06
0.05
0.04
0.03
0.02
0.01
0
C(S)
Full Genome Capsid
92S / 94%
Empty Capsid
62S / 6%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7B

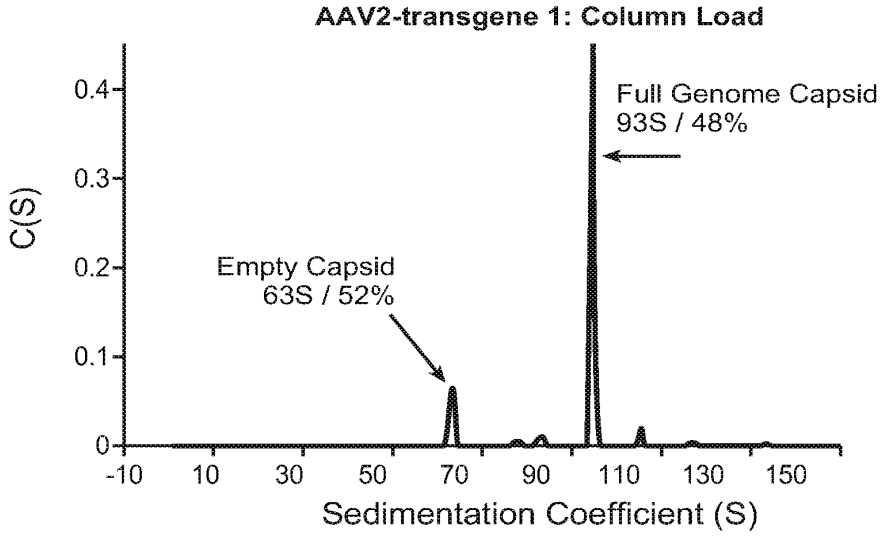

AAV2-transgene 1: Column Load
0.4
0.3
0.2
0.1
0
C(S)
Full Genome Capsid
93S / 48%
Empty Capsid
63S / 52%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7C

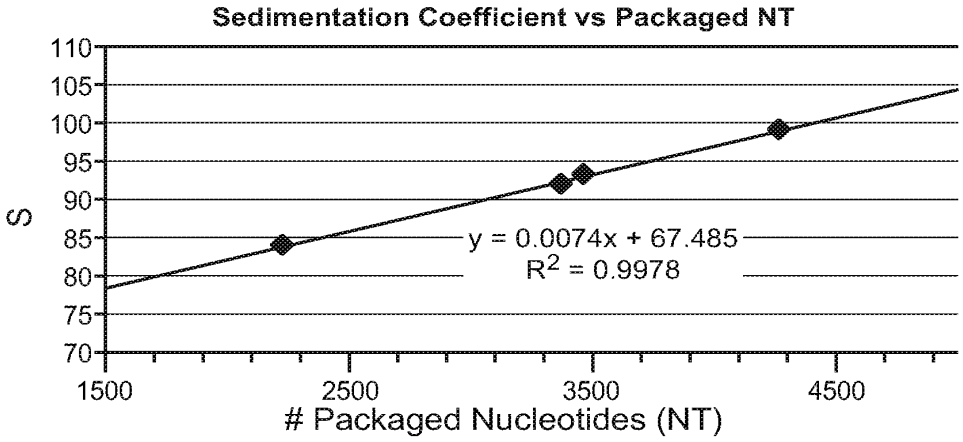

Sedimentation Coefficient vs Packaged NT
S
110
105
100
95
90
85
80
75
70
y = 0.0074x + 67.485
R2 = 0.9978
1500
2500
3500
4500
Packaged Nucleotides (NT)

FIG. 8

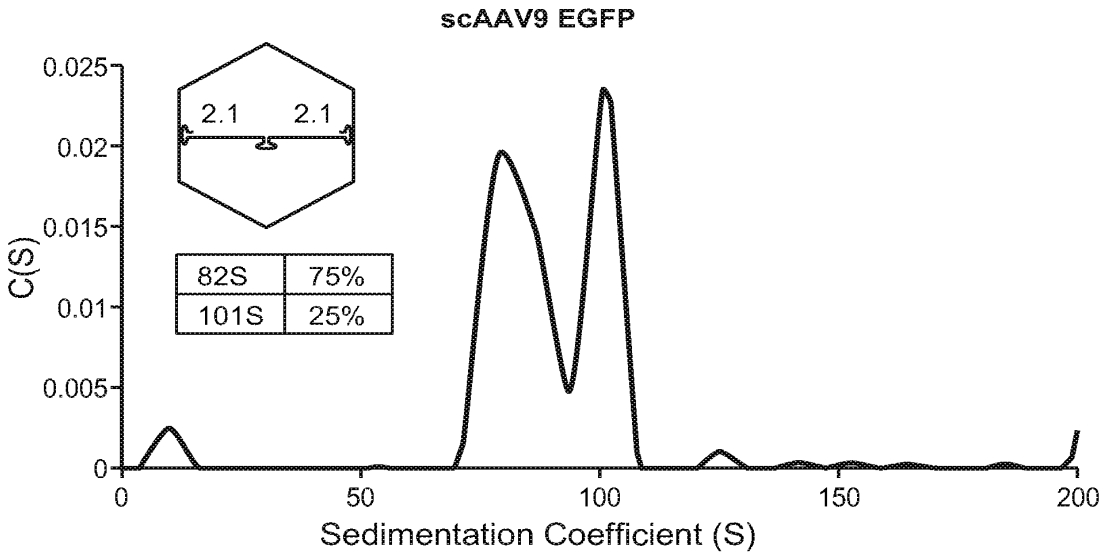

scAAV9 EGFP
C(S)
0.025
0.02
0.015
0.01
0.005
0
2.1
2.1
82S 75%
101S 25%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9A

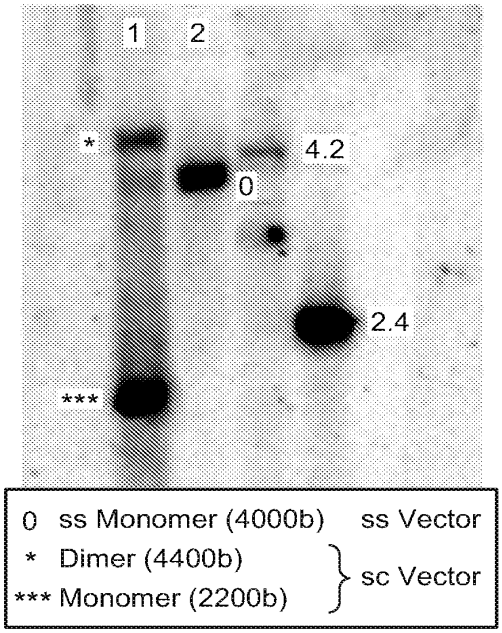

1
2
*
4.2
0
2.4
***
0 ss Monomer (4000b)
* Dimer (4400b)
*** Monomer (2200b)
ss Vector
sc Vector

FIG. 9B

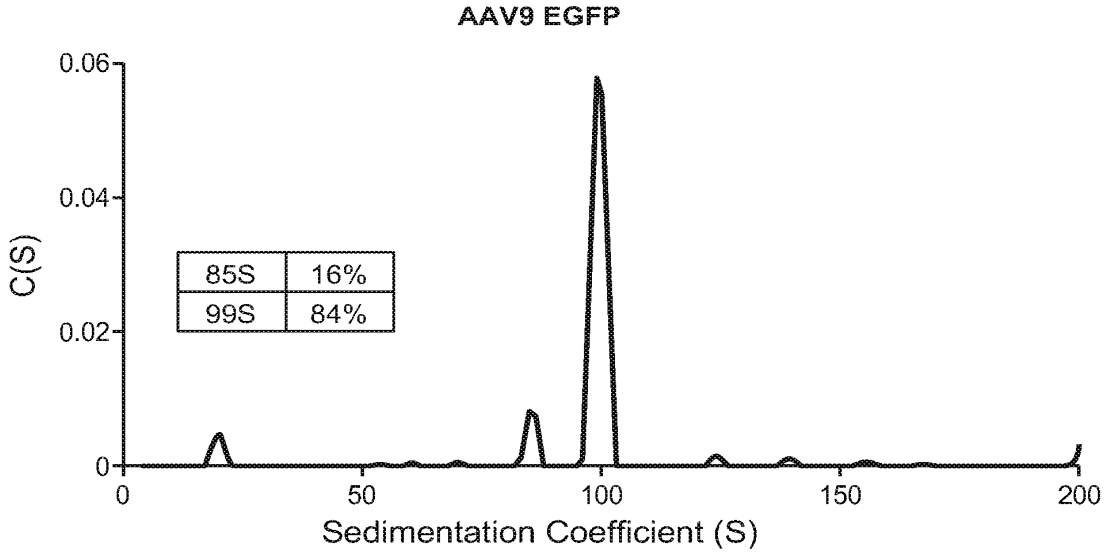

AAV9 EGFP
0.06
0.04
0.02
0
C(S)
85S 16%
99S 84%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9C

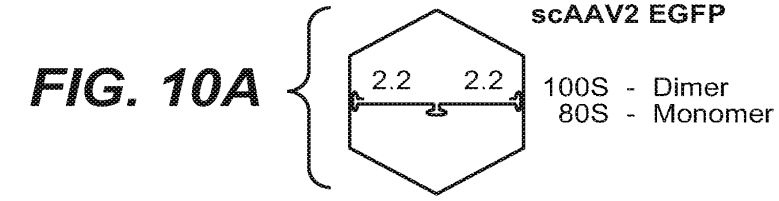


FIG. 10A

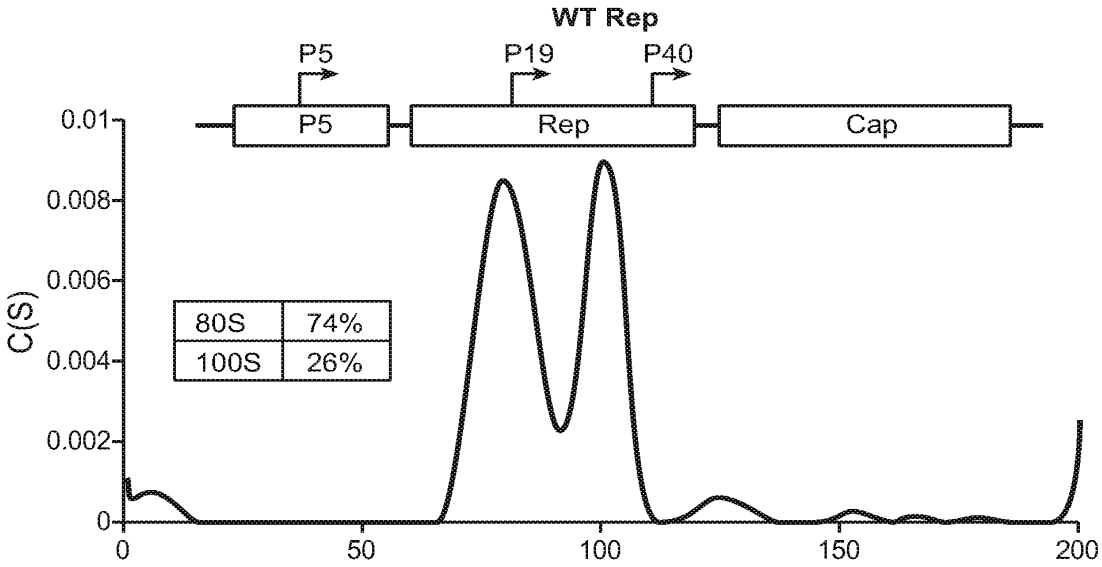


FIG. 10B

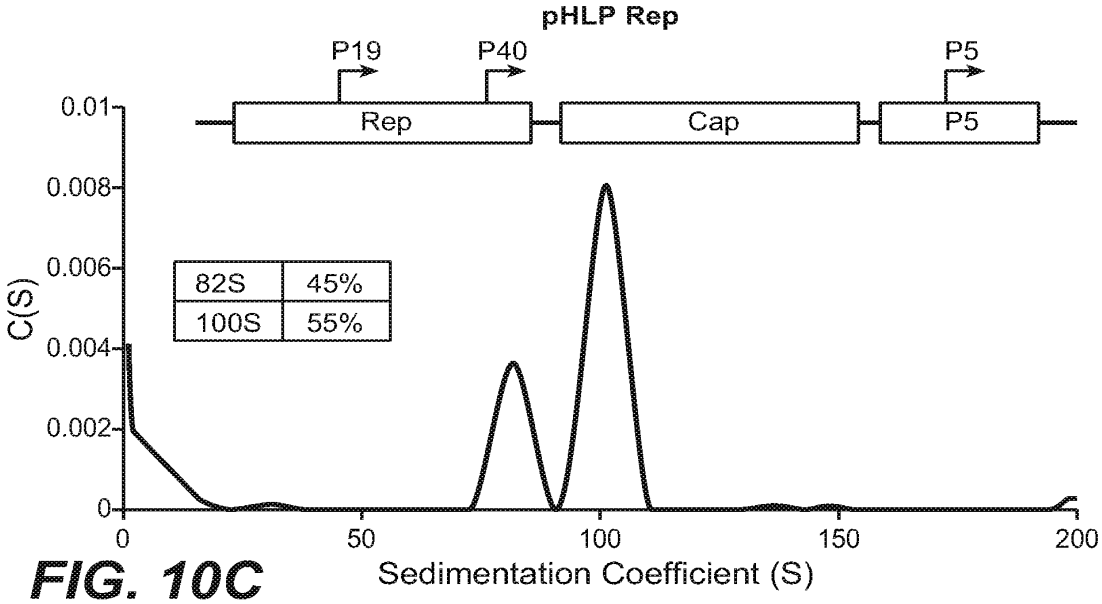


FIG. 10C

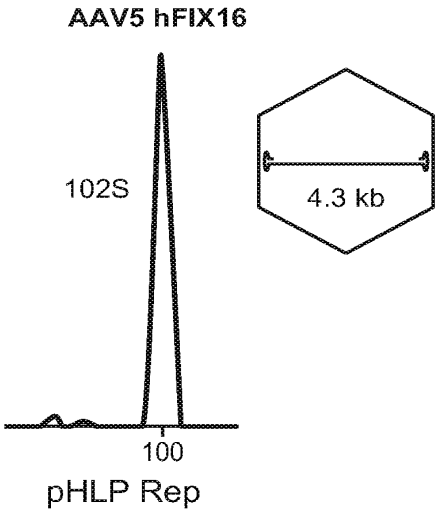


FIG. 11A

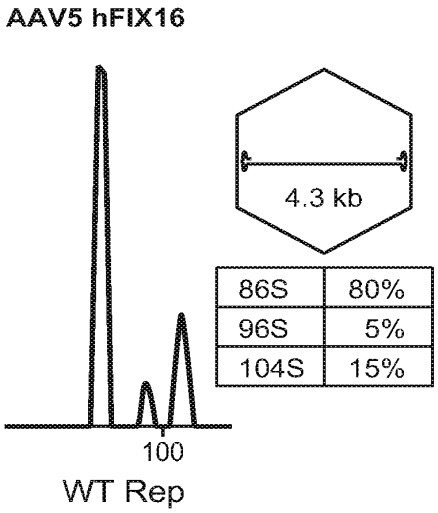


FIG. 11B

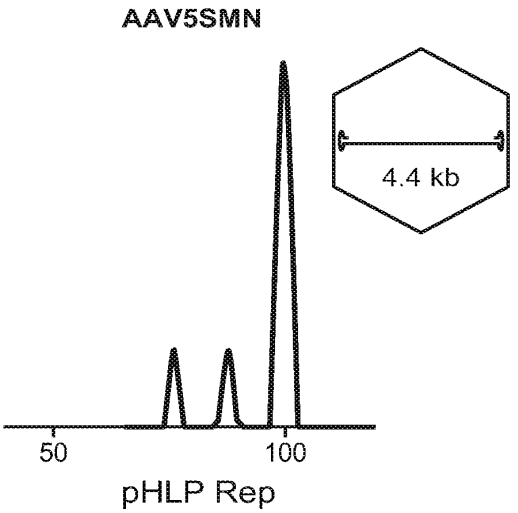


FIG. 11C

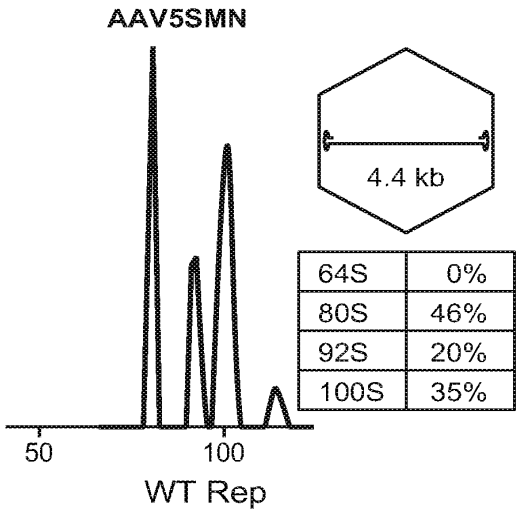
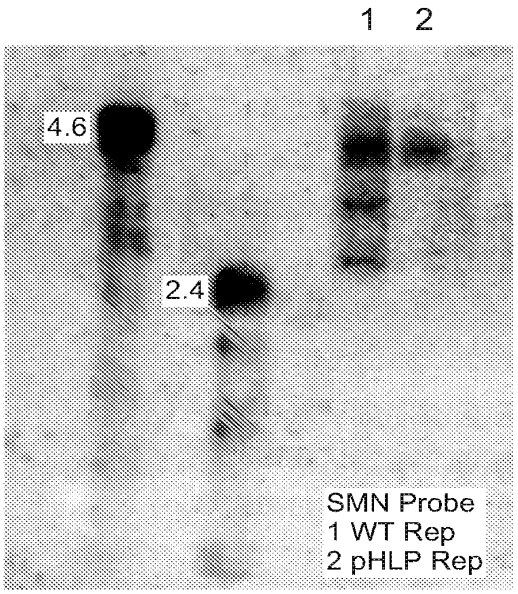
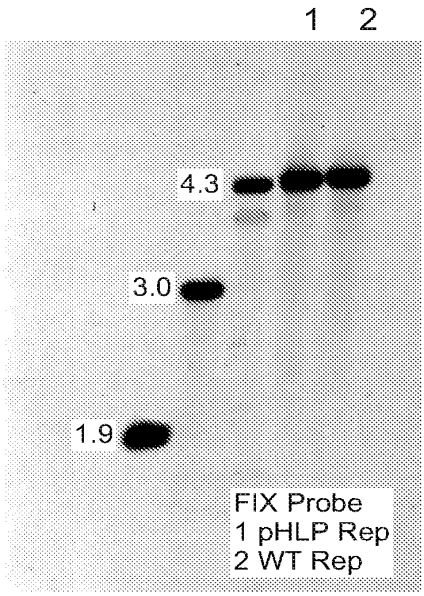


FIG. 11D



SMN Probe

FIG. 12A



FIX Probe

FIG. 12B

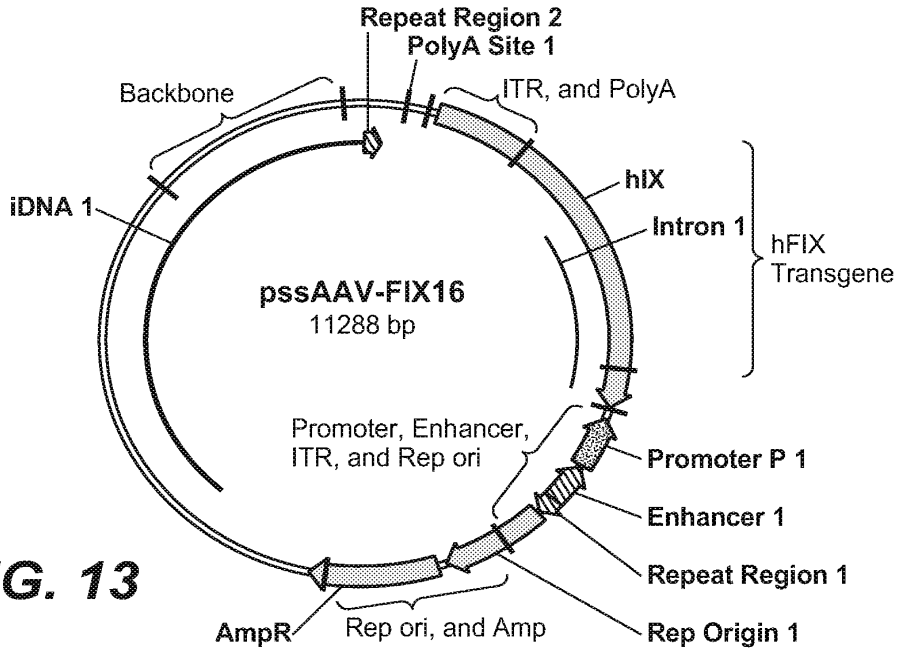
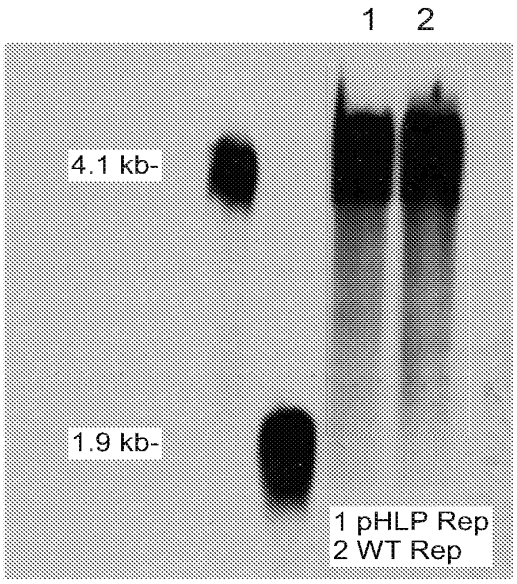
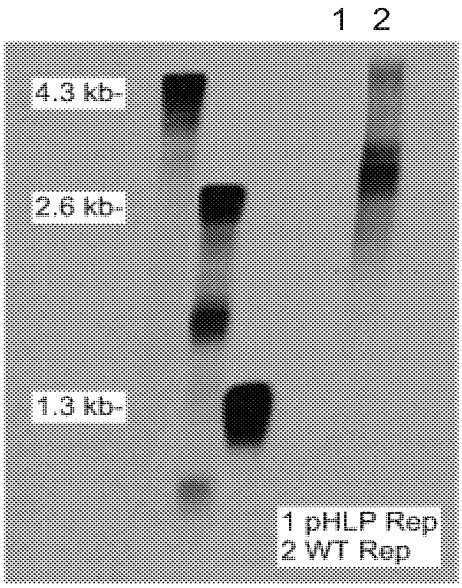


FIG. 13



Transgene

FIG. 14A



Rep ori & Amp

FIG. 14B

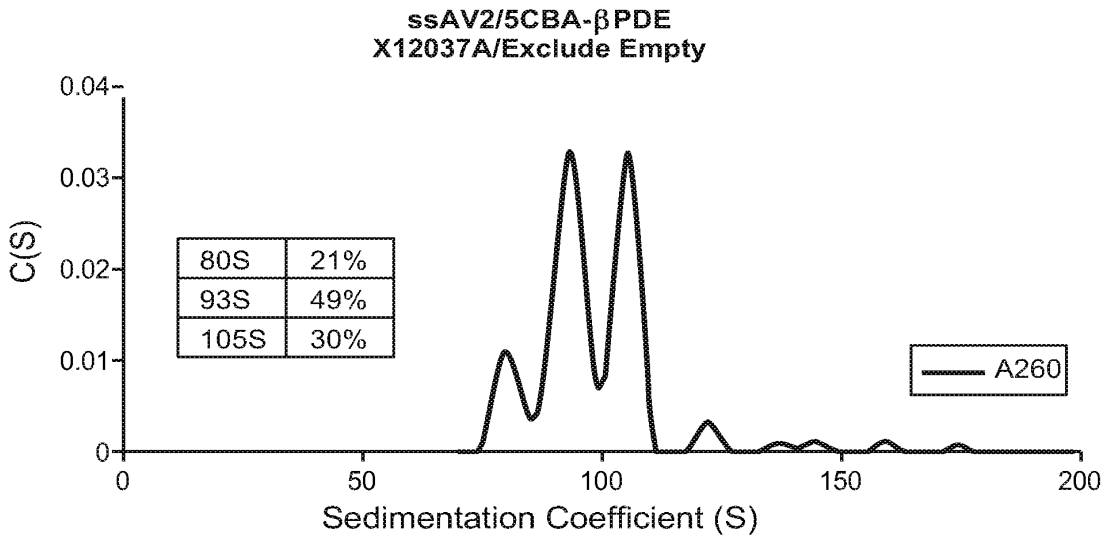


FIG. 15A

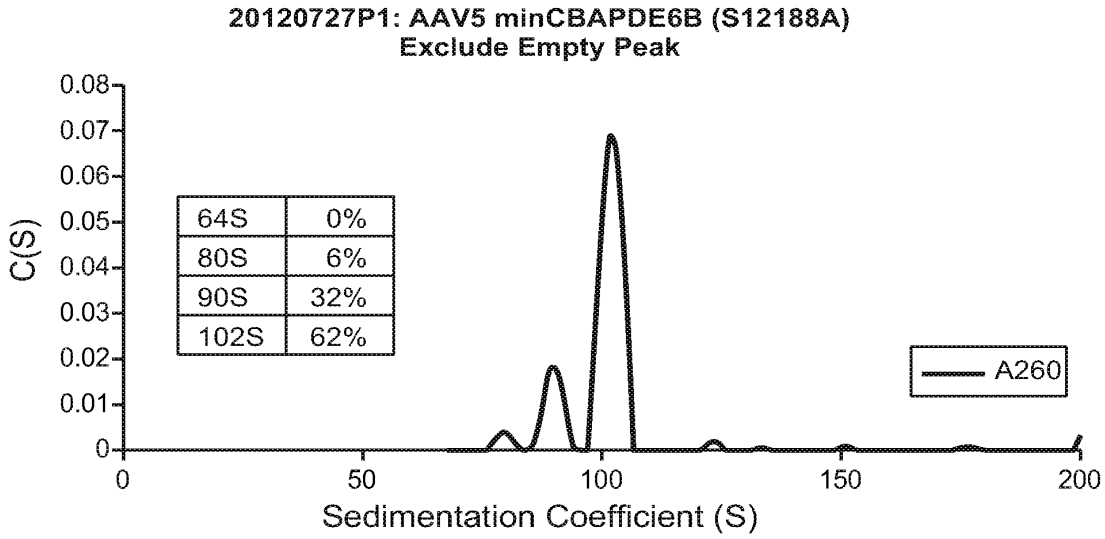


FIG. 15B

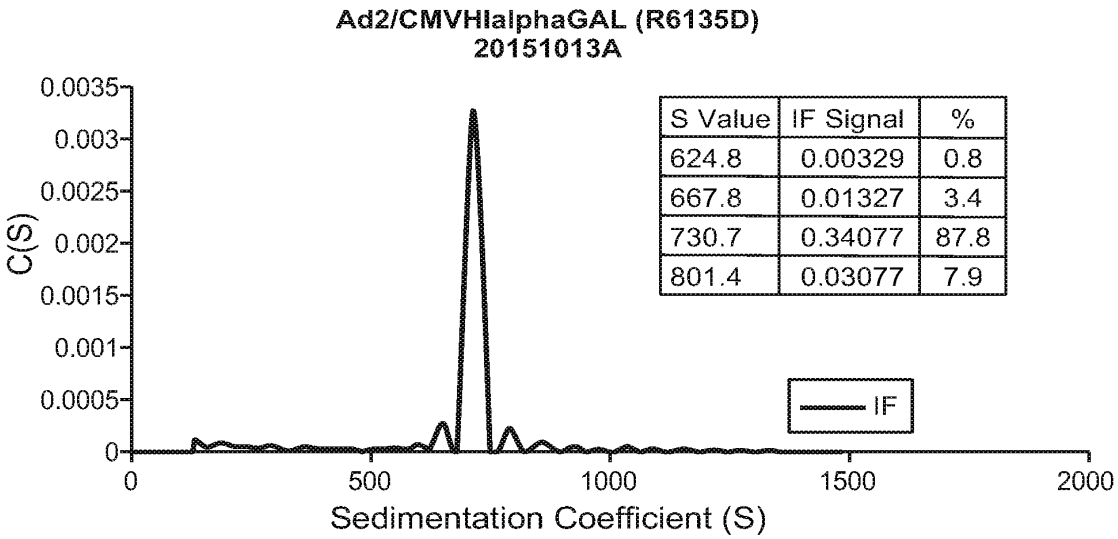

Ad2/CMVHIalphaGAL (R6135D)
20151013A
C(S)
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
500
1000
1500
2000
Sedimentation Coefficient (S)
IF
S Value | IF Signal | %
624.8 | 0.00329 | 0.8
667.8 | 0.01327 | 3.4
730.7 | 0.34077 | 87.8
801.4 | 0.03077 | 7.9

FIG. 16

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 159792010340	**FOR FURTHER ACTION** See item 4 below	
International application No. PCT/US2016/013947	International filing date *(day/month/year)* 19 January 2016 (19.01.2016)	Priority date *(day/month/year)* 20 January 2015 (20.01.2015)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant GENZYME CORPORATION		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☒	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☒	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 25 July 2017 (25.07.2017)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Agnès Wittmann-Regis e-mail: pct.team6@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

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

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

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2016/013947	19.01.2016	20.01.2015

International Patent Classification (IPC) or both national classification and IPC
INV. C12N7/02 G01N9/30

Applicant
GENZYME CORPORATION

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

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

2. **FURTHER ACTION**

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

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

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

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

Form PCT/ISA/237 (Cover Sheet) (January 2015)

Box No. I Basis of the opinion

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

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

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

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

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

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

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

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

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

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

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

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

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

5. Additional comments:

Box No. II Priority

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

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

3. Additional observations, if necessary:

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

1. Statement

Novelty (N)	Yes:	Claims	2-12, 21, 22, 24-29, 33-67, 71-95
	No:	Claims	1, 13-20, 23, 30-32, 68-70
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-95
Industrial applicability (IA)	Yes:	Claims	1-95
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

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

and / or

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

see form 210

Box No. VIII Certain observations on the international application

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

see separate sheet

1 **Re Item V**

Reasoned statement on Novelty, Inventive Step and Industrial Applicability -

The documents mentioned in the present written opinion / International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

1.1 **Novelty (Art.33(2) PCT)**

D1 discloses the assessment of the suitability of several technicques for the characterization of virus particle (in particular adenovirus) aggregation or integrity, including AUC under boundary conditions. Data analysis using SEDFIT/Lamb is shown just as in present application (see e.g. Fig.7 (p.23) - shows monomer v. dimer distribution). After assessing several techniques, the author of D1 concludes that AUC in SV mode (boundary conditions) is suitable for the assessment of aggregation, but also the integrity (incompletes, empties) of viral particles, and is capable of both identifying and quantifying such abberant particles (see Table 3). The disclosure of D1 is relatively generic in the sense that it does not disclose a number of actual methods carried out with all the steps set out in present claims, hence novelty objections are raised against independent claims 1 and 13 on the basis of the analysis shown in Fig.7. For the other independent claims novelty is provisionally acknowledged. At least dependent claims 14-20, 23, 30-32, 68-70 are also considered anticipated.

D2 discloses monitoring of the heterogeneity of adenovirus preparations using AUC under boundary conditions. The method is used to assess lot-to-lot consistency of pharmaceutical preparations. On p.17 (col.2), examples of the kind of information that can be obtained is listed. Data analysis is by SEDFIT using c(s) or ls-g(s) methods. Fig.8C shows a data analysis performed in exactly the same manner as in present application with a c(s) v S plot. In the example provided, the plot identifies various multimeric forms of adenovirus particles in a preparation. D2 is provisionally cited against the same claims as D1.

1.2 **Inventive Step (Art.33(3) PCT)**

D1 and D2 are discussed above in section 1.1. D3 is a further document applying AUC to determine the presence of empty capsids of adenovirus, in particular Ad5. The document states that AUC in CsCl density gradients should become a routine tool to evaluate the quality of rAd preparations (p.

747, top. col.2). Empty capsids were determined by absorbance measurements at 320 / 260nm at 3000 / 40000rpm. Further optimization of centrifugation parameters was performed to maximize resolution between virus particle forms with similar size densities (i.e. slight different genome size) (see p.751, col.2). Since the data was not analysed by plotting c(s) v S, D3 is not considered novelty-destroying.

Present application claims a variety of closely linked methods for assessing aspects of virus preparations:

Claim 1 - "characterizing a preparation"

Claim 2 - "assessing vector genome integrity"

Claim 4 - "determining presence empty capsids / capsid particles comprising variant sized genomes"

Claim 5 - "determining relative amount empty capsids"

Claim 6 - "measuring amount capsid particles comprising variant sized genomes / empty capsids"

Claim 7 - "measuring amount capsid particles comprising variant genomes"

Claim 8 - "measuring amount capsid particles comprising intact genomes"

Claim 13 - "determining heterogeneity of recombinant viral particles"

Due to the excessive number of alternative formulations, it is necessary to try and formulate a common problem for assessment. Applicant is considered to be addressing the problem of the provision of a method for the qualitative / quantitative assessment of particle heterogeneity (at aggregate or genome level) in viral (pref. adenoviral) preparations. Due to the inconciseness of the claims and wide variety of dependent claim parameters, these cannot be assessed on an individual level until claims are limited to specific parameters. Broadly speaking it is however considered that a skilled person starting with e.g. D1 or D2 as the closest prior art is well aware of the utility of AUC under boundary conditions for assessment of adenoviral preparations. Both D1 and D2 actually show how aggregate compositions can be effectively determined using the methodology and D1 additionally states that integrity (incompletes / empties) can also by qualitatively and quantitatively assessed by the method. This is further shown in D3, which although not applying the same algorithm to the data analysis clearly shows that the techniques resolution is sufficient for resolving even small differences in genome size (using the example of two different adenovirus vectors with genomes of slightly differing sizes). The

differences to the prior art are essentially only explicit listing of obvious features not specifically addressed but obviously within the realms of the disclosures in the prior art. These differences do not establish inventive activity. The fact that the method can achieve all that is claimed is thus immediately obvious to a skilled person viewing the prior art. Choice of exact algorithms or data analysis forms can not render the claimed subject-matter inventive, neither can the wording of an intention falling within the generic characterization of particles which is well known to be within the capabilities of the technique. Applicant is deploying known publically available software using algorithms developed by other people. He has made no contribution in this respect. Further, it is obvious that various means of tracking movement of particles in the centrifuge are available (e.g. different speeds, wavelengths etc.) or that the methodology can apply to any subtype of adenovirus particle (since the method obviously is for distinguishing particle size / density and this would not be expected to work any differently for other viruses). This also applis to the application to viruses made by different recombinant techniques. Hence, application is considered to constitute a disordered claiming of a wide variety of aspects of AUC viral particle analysis which is totally devoid of inventive subject-matter.

1.3 **Industrial Applicability (Art.33(4) PCT)**

Present claims appear to be industrially applicable.

2 **Re Item VI**

Certain documents -

In accordance with Rule 70.10, PCT, applicants attention is drawn to the following document(s):

Burnham et al. 2016 (Publication date, 12.05.2015)

3 **Re Item VIII**

3.1 **Clarity (Art.6 PCT)**

3.1.1 Present application contains 8 independent method claims essentially addressed to similar or overlapping matter worded in a number of different ways (see list in section 1.2). This number of independent claims in the same category far exceeds what can be considered acceptable in terms of conciseness.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/544,498	07/18/2017		1880	159792010300	95	10

CONFIRMATION NO. 2986

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

FILING RECEIPT

OC000000098242671

Date Mailed: 03/23/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Catherine R. O'RIORDAN, Bridgewater, NJ;
Brenda BURNHAM, Hopkinton, MA;

Applicant(s)

Genzyme Corporation, Cambridge, MA;

Assignment For Published Patent Application

Genzyme Corporation, Cambridge, MA
Genzyme Corporation, Cambridge, MA

Power of Attorney: The patent practitioners associated with Customer Number 89300

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2016/013947 01/19/2016
which claims benefit of 62/105,714 01/20/2015

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 03/22/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/544,498**

Projected Publication Date: 06/28/2018

Non-Publication Request: No

Early Publication Request: No

Title

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/544,498

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED		(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A		N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A		N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A		N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	112	minus 20 =	* 92			OR	x 100 =	9200
INDEPENDENT CLAIMS (37 CFR 1.16(h))	10	minus 3 =	* 7				x 460 =	3220
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).							400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))								820
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL			TOTAL	15220

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
15/544,498	Catherine R. O'RIORDAN	159792010300

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

INTERNATIONAL APPLICATION NO.	
PCT/US2016/013947	
I.A. FILING DATE	PRIORITY DATE
01/19/2016	01/20/2015

CONFIRMATION NO. 2986
371 FORMALITIES LETTER

OC000000098242672

Date Mailed: 03/23/2018

Notification of Insufficiency Under 37 CFR 1.492 and/or 1.495 (DO/EO/US)

The following items have been submitted by the applicant or the International Bureau to the United States Patent and Trademark Office as a Designated / Elected Office (37 CFR 1.495).

- Priority Document
- Copy of the International Application filed on 07/18/2017
- Copy of the International Search Report filed on 07/18/2017
- Preliminary Amendments filed on 07/18/2017
- Inventor's Oath or Declaration filed on 07/18/2017
- U.S. Basic National Fees filed on 07/18/2017
- Authorize Access to Search Results filed on 07/18/2017
- Priority Documents filed on 07/18/2017
- Power of Attorney filed on 07/18/2017
- Specification filed on 07/18/2017
- Claims filed on 07/18/2017
- Abstracts filed on 07/18/2017
- Drawings filed on 07/18/2017
- Authorization to Permit Access filed on 07/18/2017
- Application Data Sheet (37 CFR 1.76) filed on 07/18/2017

The following items **MUST** be furnished within the period set forth below:

- Additional claim fees of $**13240** as a undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**13240** for an Undiscounted Entity:

Total additional claim fee(s) for this application is $**13240**
- $**3220** for **7** independent claims over 3.
- $**9200** for **92** total claims over 20.
- $**820** for multiple dependent claim surcharge.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

FORM PCT/DO/EO/923 (371 Formalities Notice)

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

BRIAN A HARDEN

Telephone: (703) 756-1815

FORM PCT/DO/EO/923 (371 Formalities Notice)

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
15544498	

Applicant(s) Catherine O'RIORDAN

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
2	1					
3		1				
4	1					
5	1					
6	1					
7	1					
8	1					
9	1					
10		6				
11		6				
12		6				
13	1					
14		1				
15		1				
16		3				
17	1					
18		(1)				
19		(1)				
20		(1)				
21		(1)				
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15/544,498
	Filing Date	January 19, 2016 (Intl)
	First Named Inventor	Catherine R. O'RIORDAN
	Art Unit	1648
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	15979-20103.00

U.S. PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-6,989,264-B2		01-24-2006	Atkinson et al.	
	2.	US-8,137,948-B2		05-21-2003	Qu et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

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Examiner Initial*	Cite No	Country Code[2]	Foreign Document Number[3]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T[5]
	3.	WO-2010/148143-A1			12-23-2010	Genzyme Corporation		

NON-PATENT LITERATURE DOCUMENTS

Examiner 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.	T[5]
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EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	15979-20103.00

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2018-04-06
Name/Print	Brian Donahue	Registration Number	58,206

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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(54) **Title**: IMPROVED METHODS FOR PURIFICATION OF RECOMBINANT AAV VECTORS

(57) **Abstract**: Provided herein are methods for the purification of recombinant adeno-associated virus (rAAV) vectors that can be used for gene transfer and specifically for gene therapy or vaccination. Recombinant AAV vectors of the invention are substantially free of in-process impurities, including production components such as cellular nucleic acids, cellular proteins, helper virus, and media components.

IMPROVED METHODS FOR PURIFICATION OF RECOMBINANT AAV VECTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. provisional application Serial No. 61/187,601, filed June 16, 2009, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of purification of recombinant adeno-associated virus (rAAV) vectors that can be used for gene transfer and specifically for gene therapy or vaccination. More specifically, it relates to methods for purification of recombinant rAAV vectors that are substantially free of in-process production components such as cellular nucleic acids, cellular proteins, helper virus, and media components.

BACKGROUND OF THE INVENTION

[0003] Adeno-associated viruses (AAV) have unique features that make them attractive as vectors for gene therapy and genetics vaccines. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent, asymptomatic, and not implicated in the etiology of any human disease. Moreover, AAV infects a wide range of cell types including many mammalian cells, allowing the possibility of targeting many different tissues *in vivo*. AAV infects slowly dividing and non-dividing cells and can persist essentially for the lifetime of those cells as a transcriptionally active nuclear episome (extrachromosomal element). Integrated copies of rAAV vector in organs such as liver or muscle are very rare. Efficient long-term gene transfer has been reported in a number of cell types including eye, CNS, and muscle. *See, e.g.*, X. Xiao et al., *J. Virol.* 70(11):8098-8108 (1996); R.R. Ali et al., *Hum. Mol. Genet.* 5(5):591-94 (1996). Current clinical studies have largely focused on the use of serotype 2 rAAV vectors, but a number of reports have demonstrated that other AAV serotypes including rAAV-1, rAAV-4, rAAV-5 and rAAV-8 have unique *in vivo* bio-distribution which make them attractive viral serotypes to test in clinical trials.

[0004] Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including 145 nucleotide inverted terminal repeat (ITRs). The nucleotide sequence of the AAV serotype 2 (AAV2) genome is

presented in Srivastava et al., *J. Virol., 45*: 555-564 (1983) as corrected by Ruffing *et al., J. Gen. Virol., 75*: 3385-3392 (1994). *Cis*-acting sequences directing viral DNA replication (rep), encapsidation/packaging and host cell chromosome integration are contained within the ITRs. Three AAV promoters, p5, p19, and p40 (named for their relative map locations), drive the expression of the two AAV internal open reading frames encoding rep and cap genes. The two rep promoters (p5 and p19), coupled with the differential splicing of the single AAV intron at nucleotides 2107 and 2227, result in the production of four rep proteins (rep78, rep68, rep52, and rep40) from the rep gene. Rep proteins possess multiple enzymatic properties which are ultimately responsible for replicating the viral genome. The cap gene is expressed from the p40 promoter and it encodes the three capsid proteins VP1, VP2, and VP3. Alternative splicing and non-consensus translational start sites are responsible for the production of the three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology, 158*: 97-129 (1992).

[0005] AAV particles comprise a proteinaceous capsid having three capsid proteins, VP1, VP2 and VP3, which enclose a ~4.6 kb linear single-stranded DNA genome. Individual particles package only one DNA molecule strand, but this may be either the plus or minus strand. Particles containing either strand are infectious, and replication occurs by conversion of the parental infecting single strand to a duplex form, and subsequent amplification, from which progeny single strands are displaced and packaged into capsids. Duplex or single-strand copies of AAV genomes (sometimes referred to as "proviral DNA" or "provirus") can be inserted into bacterial plasmids or phagemids, and transfected into adenovirus-infected cells. See Carter, HANDBOOK OF PARVOVIRUSES, Vol. I, pp. 169-228 (1989), and Berns, VIROLOGY, pp. 1743-1764, Raven Press, (1990) for a general review of AAV.

[0006] rAAV vector production generally requires four common elements: 1) a permissive host cell for replication; 2) helper virus function which can be supplied by suitable helper viruses such as adenovirus or herpes virus, or alternatively by plasmid constructs containing the minimal adenoviral helper functions; 3) a trans-packaging rep-cap construct; and 4) a suitable production media.

[0007] Recombinant AAV particles can be produced from packaging cell lysates. *See, e.g.*, Chirico and Trempe (1998) *J. Virol. Methods* 76:31-41. However, the cell lysate contains various cellular components such as host cell DNA, host cell proteins, media

components and either helper virus or helper virus plasmid DNA which must be separated from the rAAV vector before it is suitable for *in vivo* use. Recent advances in rAAV production include the use of non-adherent cell suspension processes in stirred tank bioreactors and production conditions whereby rAAV vectors are released into the media or supernatant reducing the concentration of host cellular components present in the production material but still containing appreciable amounts of in-process impurities. *See* U.S. Patent No. 6,566,118 and PCT WO 99/11764. Therefore, rAAV particles may be collected from the media and/or cell lysate and further purified.

[0008] Methods including density gradient centrifugation employed for the purification of rAAV vectors and in particular rAAV-2 are not amenable to scale up. Recent reports for rAAV-2 vectors have described purification methods employing ion exchange chromatography including opposing ion exchange chromatography (including cation and anion chromatography). See for example U.S. Patent No. 6,566,118 and PCT WO 99/11764 which disclose methods of using a combination of opposing ion exchange chromatography for purifying recombinant adeno-associated virus vectors from a culture supernatant and/or a cell lysate. Additional improvements in rAAV stock preparations include the use of deoxycholate treatment of the cell lysate, iodixanol gradient separation prior to the affinity chromatography, which have resulted in high titer rAAV2 (Clark et al., *Hum. Mol. Genet.* 10(6):1031-39 (1999); Zolotukhin et al., *Gene Therapy* 6(6):973-985 (1999)). O'Riordan et al. (O'Riordan et al., *J. Gene Med.* 2:444-454 (2000); U.S. Patent No. 7,015,026) also reported scalable chromatographic purification process for recombinant adeno-associated virus vectors and as particularly exemplified, rAAV-2 vectors, using ion exchange chromatography, hydroxyapatite chromatography, cellufine sulfate affinity chromatography, and zinc chelate chromatography.

[0009] Recent data indicate that rAAV capsid serotypes such as rAAV-1, 4, 5, and 8 bind weakly to anionic resins either as purified virus stock or in the presence of in-process production impurities such as host cell DNA, host cell proteins, serum albumin, media components, and helper virus components. Consequently, purification of those capsid serotypes typically involves anion-exchange chromatography in combination with other purification methods, such as iodixinol density-gradient centrifugation. *See, e.g.*, Zolotukhin et al., Methods 28(2):158-167 (2002) and Kaludov et al., *Hum. Gene Therapy* 13:1235-1243 (2002); and U.S. Patent Publication No. 2004/0110266 A1. However, those methods are not readily scalable to commercial scale processes.

[0010] Accordingly, in the development of recombinant AAV vectors such as those for use in gene therapy and gene vaccines, there is a need for methods of purifying rAAV vectors from in-process production components including helper virus, as well as helper virus proteins, cellular proteins, host cell DNA, and media components present in the rAAV production stock. Such methods should be effectively employed on a scale that is suitable for the practical application of gene therapy techniques. Moreover there is a need for development of purification processes for rAAV vectors that are scalable to yield high titer, highly purified commercial stocks useful for rAAV gene therapy and gene vaccines. More particularly, there is a need for development of purification processes for rAAV vectors that bind weakly to chromatographic resins and in particular anionic resins.

[0011] The disclosures of all publications, patent applications, and patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. In particular, all publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methods which might be used in connection with the invention. Although the invention provided herein has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SUMMARY OF THE INVENTION

[0012] The invention provides methods of isolating a population of recombinant adeno-associated virus (rAAV) particles of any capsid serotype from in-process impurities by capturing the rAAV particles on an apatite chromatography medium in the presence of polyethylene glycol (PEG). The methods of the invention entail upstream processing (such as, for example, centrifugation, treatment with Benzonase®, anion exchange filtration, and/or tangential flow filtration) as well as downstream processing (such as, for example, heat inactivation, filtration, hydrophobic interaction chromatography, size exclusion chromatography, and/or anion exchange chromatography). The upstream and downstream methods may be used alone or in various combinations.

[0013] The invention provides methods for isolating a population of recombinant adeno-associated virus (rAAV) particles from in-process impurities in a feedstream, comprising the

steps of: (a) contacting a feedstream containing the rAAV particles with an apatite chromatography medium in the presence of polyethylene glycol (PEG), wherein the rAAV particles bind to the apatite chromatography medium; and (b) eluting the rAAV particles bound to the apatite chromatography medium with an elution buffer containing less than 3% (w/v) PEG. In certain embodiments, the apatite chromatography medium is ceramic hydroxyapatite (CHT) or ceramic fluoroapatite (CFT). In certain embodiments, the rAAV particles bound to the apatite chromatography medium are eluted with an elution buffer containing less than 3% (w/v) PEG. In certain embodiments, the rAAV particles bound to the apatite chromatography medium are eluted with an elution buffer in the absence of PEG.

[0014] In some embodiments, the specific binding of the apatite chromatography medium is between 10^6 and 10^{16} DNase-resistant particles (DRPs) per milliliter. In some embodiments, the specific binding of the apatite chromatography medium is between 10^8 and 10^{16} DNase-resistant particles (DRPs) per milliliter. In some embodiments, the specific binding of the apatite chromatography medium is between 10^{10} and 10^{16} DNase-resistant particles (DRPs) per milliliter. In some embodiments, the specific binding of the apatite chromatography medium is between 10^{12} and 10^{16} DNase-resistant particles (DRPs) per milliliter. In some embodiments, the specific binding of the apatite chromatography medium is between 10^{14} and 10^{16} DNase-resistant particles (DRPs) per milliliter.

[0015] In some embodiments, the method further comprises an anion exchange filtration step before the apatite chromatography step, wherein the rAAV particles are in the flow-through of the anion exchange filtration. In some embodiments, the method further comprises concentrating the rAAV particles from the flow-through of the anion exchange filtration by tangential flow filtration before the apatite chromatography step. In some embodiments, the method further comprises a step of binding the rAAV particles in the feedstream eluted from the apatite chromatography medium to an anionic chromatography medium. In some embodiments, the method further comprises a step of heat inactivation step to inactivate the helper virus. In some embodiments, the method further comprises a step of binding the rAAV particles in the feedstream to a hydrophobic interaction chromatography after the apatite chromatography.

[0016] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of polyethylene glycol (PEG) and a basic buffer. In some embodiments, the basic buffer is between pH 7.2 and 10, between pH 7.4 and 10, between pH 7.6 and 10, between pH 7.8 and 10, between pH 8.0 and 10.0,

between pH 8.2 and 10.0, between pH 8.4 and 10.0, between pH 8.6 and 10.0, between pH 8.8 and 10, between pH 9.0 and 10.0, between pH 9.2 and 10, between pH 9.4 and 10.0, between pH 9.6 and 10.0, or between pH 9.8 and 10.0. In some embodiments, the basic buffer has a pH of about any of 7.2, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8, 9.0, 9.2, 9.4, 9.6, 9.8, and 10.0. Any basic buffer known in the art may be used. In some embodiments, the basic buffer comprises borate. In some embodiments, the basic buffer is borate.

[0017] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of polyethylene glycol (PEG). For example, between about 3% (w/v) and about 10% (w/v) of PEG may be used. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 3% (w/v), about 3.5% (w/v), about 4% (w/v), about 4.5% (w/v), about 5% (w/v), about 5.5% (w/v), about 6% (w/v), about 6.5% (w/v), about 7% (w/v), about 7.5% (w/v), about 8% (w/v), about 8.5% (w/v), about 9% (w/v), about 9.5% (w/v), or about 10% (w/v) PEG.

[0018] In some embodiments, the PEG has an average molecular weight between about 5,000 (PEG5000) grams per mole and about 15,000 (PEG15000) grams per mole, such as, about 5,000 grams per mole (PEG5000), about 6,000 (PEG6000) grams per mole, about 7,000 (PEG7000) grams per mole, about 8,000 (PEG8000) grams per mole, about 9,000 (PEG9000) grams per mole, about 10,000 (PEG10000) grams per mole, about 11,000 (PEG11000) grams per mole, about 12,000 (PEG12000) grams per mole, about 13,000 (PEG13000) grams per mole, about 14,000 (PEG14000) grams per mole, and about 15,000 (PEG15000) grams per mole. In certain embodiments, the PEG has an average molecular weight of about 5,000 (PEG5000) grams per mole. In certain embodiments, the PEG has an average molecular weight of about 6,000 (PEG6000) grams per mole. In certain embodiments, the PEG has an average molecular weight of about 8,000 (PEG8000) grams per mole. In certain embodiments, the PEG has an average molecular weight of about 10,000 (PEG10000) grams per mole. In certain embodiments, the PEG has an average molecular weight of about 15,000 (PEG15000) grams per mole.

[0019] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of between about 3% (w/v) and about 10% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 3% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is

contacted with an apatite chromatography medium in the presence of about 4% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 5% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 6% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 7% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 8% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 9% (w/v) PEG6000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 10% (w/v) PEG6000.

[0020] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of between about 3% (w/v) and about 10% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 3% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 4% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 5% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 6% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 7% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 8% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 9% (w/v) PEG8000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 10% (w/v) PEG8000.

[0021] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of between about 3% (w/v) and

about 10% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 3% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 4% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 5% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 6% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 7% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 8% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 9% (w/v) PEG10000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 10% (w/v) PEG10000.

[0022] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of between about 3% (w/v) and about 10% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 3% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 4% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 5% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 6% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 7% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 8% (w/v) PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 9% (w/v)

PEG15000. In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in the presence of about 10% (w/v) PEG15000.

[0023] In some embodiments, the feedstream containing the rAAV particles is contacted with an apatite chromatography medium in a buffer comprising about 20 mM borate pH 9.0, and about 5% PEG (such as PEG6000). In some embodiments, the feedstream is mixed in-line with an equal volume of a buffer comprising about 40 mM borate at pH 9.0 and about 10% PEG to yield a final concentration of about 20 mM borate at pH 9.0 and about 5% PEG.

[0024] In some embodiments, the apatite chromatography medium with the rAAV particles bound to the medium is washed to remove the in-process impurities before eluting the rAAV particles. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing decreasing concentrations of PEG to remove the in-process impurities. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing between about 3% (w/v) and about 10% (w/v) PEG. In some embodiments, the wash buffer contains about any of 10% (w/v), 9.5% (w/v), 9% (w/v), 8.5% (w/v), 8% (w/v), 7.5% (w/v), 7% (w/v), 6.5% (w/v), 6% (w/v), 5.5% (w/v), 5% (w/v), 4.5% (w/v), 4% (w/v), 3.5% (w/v), and 3% (w/v) PEG. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing 7.5% (w/v) PEG6000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing 7.5% (w/v) PEG8000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing 7.5% (w/v) PEG10000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing 7.5% (w/v) PEG15000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing about 5% (w/v) PEG6000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing about 5% (w/v) PEG8000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing about 5% (w/v) PEG10000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing about 5% (w/v) PEG15000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing less than about 3% (w/v) PEG6000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing less

than about 3% (w/v) PEG8000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing less than about 3% (w/v) PEG10000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing less than about 3% (w/v) PEG15000. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing no PEG.

[0025] In some embodiments, the wash buffer contains buffers known in the art. In some embodiment, the wash buffer comprises a buffer selected from the group consisting of borate, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and Tris-HCl. In some embodiments, the wash buffer comprises or is borate. In some embodiments, the wash buffer comprises or is HEPES. In some embodiments, the wash buffer comprises or is Tris-HCl. In some embodiments, the wash buffer is at basic pH. In some embodiments, the wash buffer has a pH between pH 7.0 and pH 10.0, between pH 7.2 and pH 10.0, between pH 7.4 and pH 10.0, between pH 7.6 and pH 10.0, between pH 7.8 and pH 10.0, pH 8.0 and pH 10.0, pH 8.2 and pH 10.0, between pH 8.4 and pH 10.0, between pH 8.6 and pH 10.0, between pH 8.8 and pH 10.0, between pH 9.0 and pH 10.0, between pH 9.2 and pH 10.0, between pH 9.4 and pH 10.0, between pH 9.6 and pH 10.0, or between pH 9.8 and pH 10.0. In some embodiments, the wash buffer has a pH at 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8, 9.0, 9.2, 9.4, 9.6, 9.8, or 10.0. In some embodiments, the wash buffer comprises or is borate at a pH between 8.0 and 10.0. In some embodiments, the wash buffer comprises or is borate at pH 8.0. In some embodiments, the wash buffer comprises or is borate at pH 9.0. In some embodiments, the wash buffer comprises or is borate at pH 10.0. In some embodiments, the wash buffer comprises or is HEPES at a pH between 7.0 and 10.0. In some embodiments, the wash buffer comprises or is HEPES at pH 7.0. In some embodiments, the wash buffer comprises or is HEPES at pH 8.0. In some embodiments, the wash buffer comprises or is HEPES at pH 9.0. In some embodiments, the wash buffer comprises or is HEPES at pH 10.0. In some embodiments, the wash buffer comprises or is Tris-HCl at a pH between 7.0 and 10.0. In some embodiments, the wash buffer comprises or is Tris-HCl at pH 7.0. In some embodiments, the wash buffer comprises or is Tris-HCl at pH 8.0. In some embodiments, the wash buffer comprises or is Tris-HCl at pH 9.0. In some embodiments, the wash buffer comprises or is Tris-HCl at pH 10.0. In some embodiments, the wash buffer further comprises between 100 and 500 mM phosphate. In some embodiments, the wash buffer further comprises between 50 and 250 mM NaCl.

[0026] In some embodiments, the wash step comprises a first wash with a wash buffer comprising about 30 mM borate at pH about 9.0 and about 7.5% PEG; a second wash with a wash buffer comprising about 150 potassium phosphate, about 20 mM borate at pH about 9.0, and about 5% PEG; a third wash with a wash buffer comprising about 20 mM borate at pH about 9.0 and about 5% PEG; and a fourth wash with a wash buffer comprising about 20 mM HEPES at pH about 7.0 and 150 mM NaCl.

[0027] In some embodiments, the rAAV particles bound to the apatite chromatography medium are eluted with an elution buffer containing low concentrations of PEG or in the absence of PEG. In some embodiments, the elution buffer contains less than about 3% (w/v) PEG, less than about 2% (w/v) PEG, or less than about 1% (w/v) PEG. In some embodiments, the elution buffer contains about 2.5% (w/v), about 2% (w/v), about 1.5% (w/v), about 1% (w/v), or about 0.5% (w/v) PEG, or no PEG. In some embodiments, the elution buffer contains less than about 3% (w/v) PEG6000. In some embodiments, the elution buffer contains less than about 3% (w/v) PEG8000. In some embodiments, the elution buffer contains less than about 3% (w/v) PEG10000. In some embodiments, the elution buffer contains less than about 3% (w/v) PEG15000. In some embodiments, the rAAV particles bound to the apatite chromatography medium are eluted with an elution buffer in the absence of PEG. In some embodiments, the elution buffer comprises a buffer selected from the group consisting of borate, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and Tris-HCl. In some embodiments, the elution buffer comprises or is borate. In some embodiments, the elution buffer comprises or is HEPES. In some embodiments, the elution buffer comprises or is Tris-HCl. In some embodiments, the elution buffer is at neutral pH. In some embodiments, the elution buffer comprises or is HEPES at neutral pH. In some embodiments, the elution buffer comprises or is Tris-HCl at neutral pH. In some embodiments, the elution buffer further comprises less than 100 mM phosphate. In some embodiments, the elution buffer further comprises less than 50 mM phosphate. In some embodiments, the elution buffer further comprises between 50 and 250 mM NaCl. In some embodiments, the rAAV particles bound to the apatite chromatography medium are eluted with an elution buffer comprising about 50 mM potassium phosphate, about 20 mM HEPES at pH about 7.0, and about 150 mM NaCl.

[0028] In some embodiments, the method of isolating the rAAV particles from in-process impurities in a feedstream comprising the steps of: (a) contacting a feedstream containing the rAAV particles with an apatite chromatography medium in the presence of about 5% (w/v)

PEG in a basic buffer at pH about 9.0, wherein the rAAV particles bind to the apatite chromatography medium; (b) washing the apatite chromatography medium with a first wash buffer comprising about 30 mM borate at pH about 9.0 and about 7.5% PEG; (c) washing the apatite chromatography medium with a second wash buffer comprising about 150 potassium phosphate, about 20 mM borate at pH about 9.0, and about 5% PEG; (d) washing the apatite chromatography medium with a third wash buffer comprising about 20 mM borate at pH about 9.0 and about 5% PEG; (e) washing the apatite chromatography medium with a fourth wash buffer comprising about 20 mM HEPES at pH about 7.0 and 150 mM NaCl; and (f) eluting the rAAV particles bound to the apatite chromatography medium with an elution buffer comprising about 50 mM potassium phosphate, about 20 mM HEPES at pH about 7.0, and about 150 mM NaCl.

[0029] Also provided herein are methods for isolating a population of recombinant adeno-associated virus (rAAV) particles from in-process impurities in a feedstream, comprising the steps of: (a) contacting a feedstream containing the rAAV particles with a hydrophobic interaction chromatography (HIC) medium in a high salt buffer, wherein the rAAV particles and the in-process impurities bind the HIC medium; and (b) eluting the rAAV particles bound to the HIC medium with a medium salt buffer. In some embodiments, the HIC medium is selected from the group consisting of Tosoh Butyl 650M, Tosoh SuperButyl 650C, Tosoh Phenyl 650C, EMD Fractogel Phenyl, and Tosoh Has(butyl) resin. In some embodiments, the high salt buffer comprises or is between 0.5 M and 2.0 M citrate (e.g., sodium citrate). In some embodiments, the high salt buffer comprises about any of 0.5 M, 0.75 M, 1.0 M, 1.25 M, 1.5 M, 1.75 M, and 2.0 M citrate. In some embodiments, the medium salt buffer comprises or is less than 0.5 M citrate (e.g., sodium citrate). In some embodiments, the medium salt buffer comprising between 0.5 M to about 0.3 M citrate. In some embodiments, the medium salt buffer comprises about any of 0.45 M, 0.4 M, 0.35 M, 0.3 M, and 0.25 M of citrate. In some embodiments, the high salt buffer further comprises between 1 and 100 mM phosphate. In some embodiments, the medium salt buffer further comprises between 1 and 100 mM phosphate. In some embodiments, the medium salt buffer elutes the rAAV particles without eluting rAAV particles with empty capsids, partially denatured capsids, less infectious capsids, and/or partially full capsids.

[0030] In any of the embodiments described herein, the rAAV particles have an AAV capsid serotype selected from the group consisting of AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13, AAV-14,

AAV-15 and AAV-16. In some embodiments, the rAAV particles have an AAV capsid serotype selected from the group consisting of AAV-1, AAV-4, AAV-5, and AAV-8. In some embodiments, the rAAV particles have an AAV capsid serotype of AAV-1. In some embodiments, the rAAV particles have an AAV capsid serotype of AAV-4. In some embodiments, the rAAV particles have an AAV capsid serotype of AAV-5. In some embodiments, the rAAV particles have an AAV capsid serotype of AAV-8. In some embodiments, the rAAV particles comprise an AAV capsid protein from an AAV serotype selected from the group consisting of AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13, AAV-14, AAV-15 and AAV-16. In some embodiments, the rAAV particles have an AAV capsid serotype that is a weak anionic binder. In some embodiments, the AAV capsid serotype that is a weak anionic binding is selected from the group consisting of AAV-1, AAV-4, AAV-5, and AAV-8. In some embodiments, the composition containing rAAV particles further comprises production culture contaminants. In some embodiments, the production culture contaminants comprise damaged rAAV particles, host cell contaminants, helper virus contaminants, and/or cell culture contaminants. In some embodiments, the host cell contaminants comprise host cell DNA, plasmids, or host cell protein. In some embodiments, the helper virus contaminants comprise adenovirus particles, adenovirus DNA, or adenovirus proteins. In some embodiments, the cell culture contaminants comprise media components, serum albumin, or other serum proteins. In some embodiments, the cell culture contaminants comprise media components. In some embodiments, the cell culture contaminants do not comprise serum albumin or other serum proteins.

[0031] It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

[0032] **Figure 1** presents the results of Benzonase® digestion of the clarified supernatant from rAAV production culture harvest. The results demonstrate that no high molecular weight DNA was present following Benzonase® digestion.

[0033] **Figure 2** presents a typical spectrophotometric tracing for a typical resin screened for rAAV binding affinity as described in Example 4. Absorbance (AU) and conductivity (mS/cm) were indicated.

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[0034] **Figure 3** presents a breakthrough capacity analysis with and without PEG. Two model rAAV production cultures were used to assess capacity of the apatite resin (CFT type I). Top panel: Breakthrough during load of serum-containing or serum-free feedstreams in the presence or absence of about 5% (w/v) PEG6000 in the load. Load volumes refer to the starting feedstream, before the 1:1 online dilution, and were normalized per mL resin volume. Bottom panel: Load volumes (ml) at which 1% breakthrough was observed, and recovery in elution fraction. The TFF harvests utilized in the experiment were at a concentration of approximately 10^{16} DRP/ml for the rAAV vectors. In the presence of about 5% (w/v) PEG6000, 150 mL of the TFF harvest was loaded onto the 1.2 mL CFT resin without breakthrough, which was defined as the presence of >1% rAAV in the column flow-through, corresponding to a load of 1.8×10^{14} total rAAV DRP.

[0035] **Figure 4** presents a typical CHT I chromatogram. Shown are in-line, UV absorbance A_{280} (AU, absorbance unit) and conductivity (mS/cm) measurements by the Amersham 3 mm Skid. Brackets mark the major segments of the program described in Example 7. "NaOH" marks the column decontamination step.

[0036] **Figure 5** shows the relative purity of rAAV vectors eluted from apatite resins. Panel A shows the distribution of vector between the flow-through/chase (FT), high phosphate/5% (w/v) PEG6000 wash (PO_4), the washes to remove phosphate and PEG6000 (WII/WIII), and the elution. None of the differences between the cases are significant within the precision of the analytics, and the lack of mass balance is typical. Panel B shows the relevant lanes from a Sypro orange-stained SDS PAGE with elution fractions from the apatite column. Each sample was loaded at 2×10^{11} DRP/lane; the apparent migration difference between the lanes is a salt artifact due to having to concentrate the CFT elution by evaporation to a volume that would fit on the gel. The only predominant bands appear to be AAV capsid proteins. Panel C shows the relevant lanes of an Ad5 Western blot with lanes re-ordered for clarity, demonstrating comparable clearance of Ad5 proteins.

[0037] **Figure 6** shows assessment of purification across the process by SDS-PAGE. In-process samples from a representative production culture harvest were run on a denaturing/reducing 10% polyacrylamide gel and stained with Sypro orange. All post-harvest samples were loaded at 1×10^{10} DRP/lane. The two upstream samples before the TFF concentration step (initial clarification step and AEX flow-through) could only be loaded at 1×10^{9} DRP/lane due to volume constraints on the gel. Beta-galactosidase (B-Gal) was loaded

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at 50 ng/lane to assess sensitivity and consistency of staining across the gel. The three AAV1 capsid proteins (VP1, 2, and 3) are indicated.

[0038] **Figure 7** shows the stepwise recovery for rAAV purification as described in Examples 1–12. The total DRP present in the supernatant prior to harvest was defined as 100%. Recovery at each step is the total DRP recovered relative to total DRP processed over that step. Overall recovery for the entire process was approximately 28%. D4 sup: production culture; AEX FT: anion exchange (Mustang® Q) flow-through; capture: apatite chromatography; heat: heat inactivation or heat kill; HIC: hydrophobic interaction chromatography; SEC: size exclusion chromatography; AEX: anion exchange.

DETAILED DESCRIPTION

[0039] It is an object of this invention to provide methods for isolating a population of recombinant adeno-associated virus (rAAV) particles of any AAV capsid serotype from production culture contaminants such as damaged rAAV particles, helper virus, helper virus proteins, plasmids, cellular proteins and DNA, media components, serum proteins, and the like. Furthermore, the methods of the present invention provide commercially scalable, orthogonal processes consistent with regulatory requirements for isolation of a population of rAAV particles from high titer rAAV production culture harvests or feedstreams. The populations of rAAV particles isolated by the methods of the present invention are substantially free of contaminants, including production culture contaminants and/or in-process contaminants, such as damaged rAAV particles, helper virus, helper virus proteins, plasmids, cellular proteins and DNA, media components, serum proteins and glucans. The methods of the present invention are particularly suited to rAAV vector serotypes which are weak anionic binders such as, for example, rAAV-1, rAAV-4, rAAV-5, and rAAV-8. The invention further contemplates a method for isolating a high titer population of rAAV particles substantially free of contaminants, including production culture contaminants and/or in-process contaminants, suitable for use in gene therapy applications without the need for performing density gradient centrifugation.

Definitions

[0040] The term "isolated" or "purified" as used herein refers to a preparation of rAAV particles devoid of at least some of the other components that may also be present where the rAAV particles naturally occur or are initially prepared from. Thus, for example, isolated rAAV particles may be prepared using a purification technique to enrich it from a source

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mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like, as defined below.

[0041] A preparation of rAAV is said to be "substantially free" of helper virus if the ratio of infectious AAV particles to infectious helper virus particles is at least about 10^2:1; preferably at least about 10^4:1, more preferably at least about 10^6:1; still more preferably at least about 10^8:1. Preparations are also preferably free of equivalent amounts of helper virus proteins (*i.e.*, proteins as would be present as a result of such a level of helper virus if the helper virus particle impurities noted above were present in disrupted form). Viral and/or cellular protein contamination can generally be observed as the presence of Coomassie staining bands on SDS gels (*e.g.*, the appearance of bands other than those corresponding to the AAV capsid proteins VP1, VP2 and VP3).

[0042] The term "weak anionic binder" or "low affinity anionic binder" as used herein interchangeably refers to an rAAV particle having a capsid serotype which, in the presence of contaminants (including production culture contaminants or in-process contaminants), does not bind with sufficient affinity to allow for isolation of the rAAV particles from other rAAV production culture contaminants. Such capsid serotypes are known in the art and include, without limitation, AAV-1, AAV-5, AAV-8 and AAV-4. As described in the art, such weak anionic binders are generally purified by methods that include at least one density centrifugation step including iodixinol (sold under the trade name Optiprep®) or cesium chloride gradient centrifugation.

[0043] As used herein, the term "helper virus" or "contaminating helper virus" refers to a virus used when producing copies of a helper virus-dependent viral vector, such as adeno-associated virus, which does not have the ability to replicate on its own. The helper virus is used to co-infect cells alongside the viral vector and provides the necessary proteins for replication of the genome of the viral vector. The term encompasses intact viral particles, empty capsids, viral DNA and the like. Helper viruses commonly used to produce rAAV particles include adenovirus, herpes simplex virus, cytomegalovirus, Epstein-Barr virus, and vaccinia virus.

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[0044] The term "production culture" as used herein refers to a vessel containing the necessary components for rAAV vector particle production. Production cultures include, without limitation, the following components: 1) a suitable host cell; 2) helper virus function; 3) AAV rep and cap genes and gene products; 4) the therapeutic transgene flanked by AAV ITR sequences; and 5) suitable media, media components, and media supplements, including without limitation serum, serum-derived proteins, vitamins, essential and non-essential amino acids, and glucose known to support rAAV production.

[0045] As used herein, the terms "contaminants," "production culture contaminants," "in-process contaminants," "in-process impurities," "impurities," or "contaminants," as used interchangeably herein, refer to, without limitation, media formulations known in the art to support production of rAAV vectors; media supplements such as salts, calf serum, amino acid supplements, vitamin supplements, growth factors, serum albumin and other low molecular weight proteins present in media formulations know in the art; permissive host cells, host cell proteins or host cell DNA; helper viruses, helper virus proteins, or helper virus DNA such as wild type adenovirus or herpes virus proteins; and other non rAAV vector or rAAV vector production culture materials introduced during the purification process such as glucans or chromatography buffers utilized in the purification of rAAV vectors from feedstreams.

[0046] The term "production culture harvest" as used herein is defined as a solution comprising rAAV vector particles produced from rAAV vector production cultures by means known in the art, including without limitation transfection processes, stable cell line production, Ad-hybrid production systems, or baculovirus production systems. Furthermore, the term "production culture harvest" as used herein refers to the material isolated from the production culture vessel and includes both materials isolated by lysis of rAAV producer cells by means known in the art and materials isolated from rAAV production cultures maintained under culture conditions known in the art to yield rAAV particles released into the media from intact cells. A production culture harvest may contain some or all of the following, without limitation: rAAV vector particles, production culture components, such as, media components, host cell proteins, host cell DNA, host cells, helper virus, helper virus proteins, helper virus DNA, plasmid DNA, carrier virus DNA, serum, serum-derived proteins and media supplements.

[0047] The term "feedstream" as used herein refers to a source of rAAV vector particles that is loaded onto, passed through, or applied to a chromatographic matrix. Feedstreams of the present invention include production culture harvests, and materials isolated from

previous chromatographic steps of the invention whether the material was present as flow-through from the previous step, bound and eluted in the previous step, present in the void volume of the previous step or present in any fraction obtained during the purification of rAAV particles. Such feedstreams may include one or more "contaminants," "production culture contaminants," "in-process contaminants," "in-process impurities," or "impurities," or "contaminants," as defined herein.

[0048] The terms "capture," "bound," "binds," or "binding" as used herein interchangeably refer to the binding, adherence or sticking of a component of a feedstream to a chromatographic medium. Components may be bound to a chromatographic medium by any force or chemistry known in the art, including without limitation hydrophobic, ionic (including anionic and cationic), affinity, metal chelation, and chelation. Components may be bound to a chromatographic medium by more than one type of chemistry such as in apatite chromatographic media.

[0049] The terms "apatite resin," "apatite chromatographic medium," "apatite matrix" or "apatite medium," as used herein interchangeably refer to a chromatographic medium comprised of a mineral of calcium phosphate, and includes without limitation ceramic hydroxyapatite (CHT) and ceramic fluoroapatite (CFT) chromatographic media.

[0050] The terms "mixed mode" or "multimodal" refer to chromatographic media which have the capacity for more than one binding chemistry. Mixed mode chromatographic media include without limitation apatite chromatographic media, which are capable of exhibiting metal affinity binding via the calcium moieties, hydrogen bonding via the hydroxyl groups present on the backbone, positive charge repulsion and negative charge attraction via the calcium moieties and negative charge repulsion and positive charge attraction via the phosphate moieties present on the media.

[0051] General reference to "the composition" or "compositions" includes and is applicable to compositions of the invention.

[0052] As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise. For example, the phrase "a virus particle" includes one or more virus particles.

[0053] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X."

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[0054] It is understood that aspects and embodiments of the invention described herein include consisting and/or consisting essentially of aspects and embodiments.

Production of rAAV Vectors

[0055] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include Adenovirus-AAV hybrids, herpesvirus-AAV hybrids and baculovirus-AAV hybrids. rAAV production cultures for the production of rAAV virus particles all require; 1) suitable host cells, including, for example, human-derived cell lines such as HeLa, A549, or 293 cells, or insect-derived cell lines such as SF-9, in the case of baculovirus production systems; 2) suitable helper virus function, provided by wild type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus, baculovirus, or a plasmid construct providing helper functions; 3) AAV rep and cap genes and gene products; 4) a transgene (such as a therapeutic transgene) flanked by AAV ITR sequences ; and 5) suitable media and media components to support rAAV production. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors.

[0056] Suitable rAAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, rAAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of rAAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of rAAV in production cultures.

[0057] rAAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, rAAV production cultures include

attachment-dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. rAAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF- 9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0058] rAAV vector particles of the invention may be harvested from rAAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of rAAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

Purification of rAAV Vectors

[0059] At harvest, rAAV production cultures of the present invention may contain one or more of the following: (1) host cell proteins; (2) host cell DNA; (3) plasmid DNA; (4) helper virus; (5) helper virus proteins; (6) helper virus DNA; and (7) media components including, for example, serum proteins, amino acids, transferrins and other low molecular weight proteins. In addition, rAAV production cultures further include rAAV particles having an AAV capsid serotype selected from the group consisting of AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13, AAV-14, AAV-15 and AAV-16. In some embodiments, the rAAV particles have an AAV capsid serotype selected from the group consisting of AAV-1, AAV-4, AAV-5, and AAV-8.

[0060] In some embodiments, the rAAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade D0HC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL10 Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0061] In some embodiments, the rAAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In

some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37ºC for a period of 30 minutes to several hours.

[0062] rAAV particles may be isolated or purified using one or more of the following purification steps: flow-through anionic exchange filtration, tangential flow filtration (TFF) for concentrating the rAAV particles, rAAV capture by apatite chromatography, heat inactivation of helper virus, rAAV capture by hydrophobic interaction chromatography, buffer exchange by size exclusion chromatography (SEC), nanofiltration, and rAAV capture by anionic exchange chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below.

Anionic Exchange Filtration

[0063] Optionally in some embodiments, the clarified and Benzonase®-treated production culture harvest is subjected to anionic exchange filtration under conditions where the rAAV vector is present in the flow-through and contaminating helper virus is retained on the charged filter. At the ionic strength of the rAAV production culture harvest, the rAAV particles can be distinguished from helper virus, for example, adenovirus by passage through an anionic filter such as a Mustang® Q filter (Pall Corp., East Hills, NY). One skilled in the art can determine the size and number of filters necessary to achieve the optimal log reduction of adenovirus (LRV) and adenoviral proteins present in the clarified, Benzonase®-treated and anionic filtered production culture. In some embodiments, the LRV is at least one log and greater than ten logs. In a preferred embodiment, the LRV is at least two and greater than eight logs. In a more preferred embodiment the LRV is at least six logs.

Tangential Flow Filtration (TFF) Concentration

[0064] In some embodiments, the flow-through from the anionic filtration of the clarified, Benzonase®-treated feedstream is concentrated via tangential flow filtration ("TFF") before being applied to an apatite chromatographic medium. Large scale concentration of viruses using TFF ultrafiltration has been described by R. Paul et al., HUMAN GENE THERAPY, 4:609-615 (1993). TFF concentration of the feedstream enables a technically manageable volume of feedstream to be subjected to the chromatography steps of the present invention and allows for more reasonable sizing of columns without the need for lengthy recirculation times. In some embodiments, the rAAV feedstream is concentrated between at least two-fold and at least ten-fold. In some embodiments, the feedstream is concentrated between at least ten-fold

and at least twenty-fold. In some embodiments, the feedstream is concentrated between at least twenty-fold and at least fifty-fold. One of ordinary skill in the art will also recognize that TFF can also be used at any step in the purification process where it is desirable to exchange buffers before performing the next step in the purification process.

rAAV Capture by Apatite Chromatography in the Presence of Polyethylene Glycol (PEG)

[0065] FDA-approved processes for purification of proteins and other biologic products suitable for use in human clinical trials and pharmaceutical products rely upon commercial scale orthogonal processes. A multi-step purification scheme is considered to include an orthogonal process if it employs separation mechanisms that are distinct from one another, with each step representing an axis in Cartesian space. For example, a two-step process using anion exchange and hydrophobic interaction chromatography (HIC) would be considered orthogonal. The processes for removing contaminants, such as production culture contaminants or in-process contaminants, from a production culture harvest or feedstream described herein are orthogonal processes including both capture and flow-through steps on a variety of chromatographic media for the final product (*i.e.*, an rAAV vector). rAAV vectors (specifically rAAV-2) have been demonstrated in the art to bind anionic resins. rAAV vectors such as rAAV-1, -5, and -8 have been demonstrated to bind much less tightly than rAAV-2 to anionic exchange media in the presence of production components such as serum albumin, helper virus components, production media components and host cell DNA, resulting in a less efficient and lower quality purification scheme.

[0066] Previous purification strategies described in the art for lower affinity anionic binders such as AAV-1 included an iodixinol step gradient which reduces the relative concentration of the contaminants, such as production culture contaminants and in-process impurities, in order to achieve a tighter binding of the rAAV vector to anionic exchangers. Iodixinol step gradients are not readily scalable to commercial scale processes described herein.

[0067] The inventors of the present application have discovered that rAAV vector particles can be isolated from contaminants, such as production culture contaminants or in-process contaminants, by capture and elution from apatite resins. Thus, in addition to capturing product from a crude feedstream, the apatite column clears a variety of process-related impurities, including host cell and adenovirus proteins, glucans, and serum proteins, as well as providing additional clearance factors for helper virus (such as Ad5 helper virus).

[0068] Apatite resins are chromatography media comprising minerals of calcium phosphate, including without limitation ceramic hydroxyapatite (CHT) and ceramic fluoroapatite (CFT). Apatite chromatographic media are also referred to as mixed mode or multi-mode media because apatite has functional groups that provide more than one binding chemistry. Without wishing to be bound by theory, apatite media provide the opportunity for calcium metal affinity binding, hydrogen bonding, positive charge repulsion, positive charge attraction, negative charge repulsion, and negative charge attraction via a host of different chemical groups, including hydroxyl residues present on the backbone, positively charged calcium moieties and negatively charged phosphate moieties present on the resin. Each binding chemistry applies to mixed mode binding just as it does for single mode chromatography. However, unlike in single mode chromatography, the various binding and elution chemistries are not independent and can work in opposing ways. For example, increasing the ionic strength can drive hydrophobic binding. (T. Kawasaki, M. Niikura, and Y. Kobayashi, *J. Chrom.* 515:125–148 (1990) and P.S. Gagnon, P. Ng, J. Zhen, C. Aberin, and J. He, *BioProcess Int'l* 4:50–60 (2006)). Specifically, CHT and CFT are spherical, macroporous forms of hydroxyapatite $(Ca_5(PO_4)_3OH)_2$ sintered at high temperatures to convert the mineral from a crystalline to a ceramic form. This yields a chromatography medium with a macroporous structure providing a large surface area, limited mass-transfer resistance, high mechanical strength, and base resistance. Sintering at different temperatures and times results in different physical structures–types I and II–which are identical chemically but offer different capacities for different classes of molecules. CFT differs from CHT in that it is a composite of fluoroapatite and hydroxyapatite prepared by chemically replacing the hydroxyl groups with fluorine groups to increase stability to acidic conditions. CFT and CHT resins are commercially available (e.g., from Bio-Rad Laboratories, Inc.).

[0069] The inventors of the present invention have surprisingly discovered that the presence of polyethylene glycol (PEG) in the loading buffer dramatically increases the capacity and reproducibility (by reducing the variable breakthrough of rAAV particles in the flow-through) of rAAV vector particle binding to apatite resins. Without wishing to be bound by theory, one attribute of rAAV vectors which distinguishes them from the majority of process-related impurities is the large physical size of the particles. This size differential was exploited in the capture and wash steps by including polyethylene glycol (PEG) in the chromatography binding and wash buffers to preferentially increase the partitioning coefficients of larger molecules to the bound state based on energetically favorable sharing of

hydration shells. While use of PEG in purifying viral and bacteriophage vectors has been described in the art, unlike the present invention, it was used primarily as a precipitating agent to physically aggregate and remove viral particles from solution. Since PEG is known in the art to facilitate aggregation and precipitation of viral particles and rAAV has been described in the art to form aggregates at ionic strength below 200mM (Wright et al., *Molecular Therapy* 12:171-178 (2005)), the effect of PEG on rAAV vector binding to apatite resins was unpredictable. PEG was known in the art to facilitate binding of immunoglobulin molecules to ion exchange resins as described, for example, in Gagnon, *J. Chromtogr.* 743A:51-55 (1996), and for charged hydrophobic mixed mode resins as described, for example, in Gagnon et al., *22nd International IBC Conference on Antibody Production and Development*, March 4-6, 2009.

[0070] The inventors of the present application have determined based on experimentation with PEG6000 over a concentration range between 3-10% (w/v) in the feedstream that a relative concentration of about 5% (w/v) PEG6000 was optimal. One of ordinary skill in the art will appreciate that other species and molecular weights of PEG can be utilized including without limitation PEG8000, PEG10000, and PEG15000, and that the relative concentration of PEG at the final concentration in the rAAV vector solution can be empirically determined such that at the appropriate concentration of PEG, rAAV vector particles in the solution are driven to bind to the apatite resin but do not form aggregates or physically precipitate.

[0071] In some embodiments, the rAAV vector particles are isolated from production culture contaminants by capture on an apatite resin in the presence of PEG and elution of the bound rAAV particle from the apatite resin in a phosphate buffer. In preferred embodiments, the rAAV vector particles are isolated from production culture contaminants by capture on an apatite resin in the presence of PEG and elution of the bound rAAV particle from the apatite resin in a buffer in the absence of PEG. In some embodiments, rAAV particles comprising capsids that are weak anionic binders are isolated from production culture contaminants by capture on an apatite resin in the presence of PEG and the bound rAAV particle eluted of from the apatite resin in a buffer in the absence of PEG. In more preferred embodiments, rAAV particles comprising capsids of serotype 1 (rAAV-1 serotype) are isolated from production culture contaminants by capture on an apatite resin in the presence of PEG and the rAAV-1 serotype capsid containing particles bound to the resin are eluted in buffer in the absence of PEG. In some embodiments, the rAAV vectors particles are isolated from

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production culture contaminants according to a method comprising loading a feedstream in a loading buffer in the absence of phosphate but in the presence of PEG and eluting the bound rAAV from the apatite resin in an elution buffer comprising phosphate and lacking PEG.

[0072] While apatite chromatography in the presence of PEG represents an efficient capture or binding strategy for purification of rAAV vectors, many in-process impurities were also retained by the apatite resin at pH 7.0. Without wishing to be bound by theory, proteins present in the feedstream at basic pH (pH greater than 7.0) would be more likely to have a net negative charge and be repelled by the negative phosphate binding sites present on the apatite resin, thereby reducing the overall cation exchange binding capacity of the chromatographic resin. Given the mixed mode nature of apatite resins, however, binding via positive charge attraction and metal affinity could still occur.

[0073] Borate buffers are routinely used in the art as basic buffering systems because of their desirable manufacturing properties including without limitation ease of preparation, optimal solubility, excellent buffering capacity, and low cost. Therefore borate buffers as the model basic buffering system were evaluated for rAAV capture on apatite resins. One of ordinary skill in the art can appreciate that other basic buffers could be evaluated to determine if they reduced the level of in-process impurity binding to apatite resins in the presence of PEG. Other basic buffers may be tested and use for rAAV capture. In some embodiments, the apatite loading buffer in the absence of PEG comprises a borate buffer. In preferred embodiments, the borate buffer is formulated at a pH between about 8.0 to about pH 9.9. In a preferred embodiment, the borate buffer is formulated at a pH of about 9.0. In some embodiment, the borate buffer is at a concentration between about 5mM to about 500mM. In more preferred embodiments, the borate buffer is formulated at about 20mM borate, pH 9.0. In some embodiments, the 20 mM borate buffer at pH 9.0 specifically reduces the capture of small molecule in-process impurities on the apatite resin.

[0074] In some embodiments, the feedstream is loaded onto the apatite resin in a buffer containing phosphate in the presence of PEG by online mixing of the feedstream with a phosphate buffer comprising PEG at twice the final concentration of PEG. In some embodiments, the pH of the phosphate buffer is between pH 6.5 and pH 7.0. In some embodiments, the PEG is PEG6000. In some embodiments, the concentration of PEG6000 in the loading buffer is between about 3% (w/v) and about 10% (w/v). In more preferred embodiments, the concentration of PEG6000 in the loading buffer is about 5% (w/v). In

some embodiments, the concentration of phosphate in the loading buffer for the apatite resin is between 5mM and 500mM

[0075] In some embodiments, the binding capacity of the apatite resin in the presence of PEG is enhanced relative to the binding capacity of the apatite resin in the absence of PEG. In some embodiments, the binding capacity of the apatite resin for rAAV vector particles in a feedstream in the presence of PEG is enhanced from about one-half of a log to about ten logs relative to the binding capacity of the apatite resin in the absence of PEG. In preferred embodiments, the binding capacity of the apatite resin for rAAV particles present in a feedstream in the presence of PEG is enhanced eight logs. In some embodiments, the binding capacity of the apatite resin for rAAV vector particles in a feedstream in the presence of PEG is at least about 10^6 particles of rAAV per ml of resin to about 10^{16} particles per ml of resin (such as about any of 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15}, 10^{16} particles per ml of resin). In some embodiments, the binding capacity of the apatite resin in the presence of PEG is about 10^{14} particles per ml of resin.

[0076] While this surprising binding capacity of approximately 10^{12}-10^{14} DRP of rAAV-1 per ml of apatite resin in the presence of PEG allows for highly efficient, cost-effective scaling of commercial rAAV-1 purification, one of ordinary skill in the art will appreciate that the binding capacity represents the maximum number or rAAV-1 that will bind per ml of resin and is not intended to operationally limit the scope of the invention. Indeed the inventors appreciate that rAAV-1 vector harvest cultures that contain less than 10^{14}-10^{16} DRP of rAAV-1/ml may be purified by the present invention.

[0077] In some embodiments, the rAAV particles bound to the apatite medium is washed before eluting the rAAV particles from the resin. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing decreasing concentrations of PEG to remove the in-process impurities. In some embodiments, the apatite chromatography medium is washed one or more times with a wash buffer containing between about 3% (w/v) and about 10% (w/v) PEG. In some embodiments, the wash buffer contains about any of 10% (w/v), 9.5% (w/v), 9% (w/v), 8.5% (w/v), 8% (w/v), 7.5% (w/v), 7% (w/v), 6.5% (w/v), 6% (w/v), 5.5% (w/v), 5% (w/v), 4.5% (w/v), 4% (w/v), 3.5% (w/v), and 3% (w/v) PEG. In some embodiments, the apatite medium is washed with a wash buffer containing PEG at a concentration higher than the PEG concentration used for allowing binding of the rAAV particles to the apatite medium. In some embodiments, the apatite medium is further washed with decreasing concentration of PEG. In some

embodiments, the wash buffer contains buffers known in the art. In some embodiment, the wash buffer comprises a buffer selected from the group consisting of borate, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and Tris-HCl. In some embodiments, the wash buffer is at basic pH. In some embodiments, the wash buffer has a pH between pH 7.0 and pH 10.0, between pH 7.2 and pH 10.0, between pH 7.4 and pH 10.0, between pH 7.6 and pH 10.0, between pH 7.8 and pH 10.0, pH 8.0 and pH 10.0, pH 8.2 and pH 10.0, between pH 8.4 and pH 10.0, between pH 8.6 and pH 10.0, between pH 8.8 and pH 10.0, between pH 9.0 and pH 10.0, between pH 9.2 and pH 10.0, between pH 9.4 and pH 10.0, between pH 9.6 and pH 10.0, or between pH 9.8 and pH 10.0. In some embodiments, the wash buffer has a pH at 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8, 9.0, 9.2, 9.4, 9.6, 9.8, or 10.0. In some embodiments, the wash buffer further comprises between 100 and 500 mM phosphate. In some embodiments, the wash buffer further comprises between 50 and 250 mM NaCl.

[0078] In some embodiments, the rAAV vectors isolated from a feedstream by capture on an apatite resin in the presence of PEG are eluted in a buffer in low concentrations of PEG. In some embodiments, low concentrations of PEG are between about 2.9% (w/v) and about 0.1% (w/v) PEG. In some embodiments, the rAAV vectors isolated from a feedstream by capture on an apatite resin in the presence of PEG are eluted in a buffer in the absence of PEG. In preferred embodiments, the rAAV vectors isolated from a feedstream by capture on an apatite resin in the presence of PEG are eluted in a buffer containing phosphate in the absence of PEG.

[0079] In some embodiments, the rAAV vectors isolated from a feedstream by capture on an apatite resin in the presence of PEG are eluted in a buffer containing phosphate. In some embodiments, the rAAV vectors isolated from a feedstream by capture on an apatite resin in the presence of PEG are eluted in a buffer containing phosphate at a concentration between about 0.1 mM to about 500 mM (such as, between about 1 mM to about 250 mM, between about 10 mM to about 100 mM). In preferred embodiments, the rAAV vectors isolated from a feedstream by capture on an apatite resin in the presence of PEG are eluted in a 50mM phosphate buffer.

[0080] The inventors of the present application have discovered that rAAV vectors present in a feedstream can be isolated by capture on an apatite resin in the presence of PEG. However if helper viruses used in the production culture (such as adenovirus) are present in the feedstream applied to the apatite resin, they are captured by the apatite resin in the

presence of PEG. rAAV vector particles captured by the apatite resin in the presence of PEG can be easily isolated from adenovirus by their elution profile in phosphate buffers. rAAV vector particles bound to the apatite resin in the presence of PEG, elute, in the absence of PEG, in buffers containing as low as 0 mM phosphate; whereas helper virus adenoviral particles are retained on the apatite resins under concentrations of phosphate used to elute the rAAV vector particles. Experimentally, rAAV vectors were found to elute in a single sharp peak in as little as 50 mM phosphate in the absence of PEG, whereas helper virus such as adenovirus if present were retained on the resin. In spike-in studies in which rAAV feedstreams were spiked with 8^9 DNase-resistant particles (DRPs) of infectious adenovirus and subjected to chromatography on apatite resins in the presence of PEG, the rAAV vectors were captured on the apatite resin and eluted in 50 mM phosphate buffer in the absence of PEG, while approximately 4 logs of adenoviral proteins were retained on the apatite resin. Accordingly, in some embodiments, the rAAV vectors present in a feedstream are isolated from contaminating helper virus by capture on an apatite resin in the presence of PEG and elution in phosphate buffers in the absence of PEG. In some embodiments, the phosphate buffers are formulated at concentrations that retain contaminating helper virus bound to the apatite resin. In some embodiments, two to eight logs of adenovirus are retained per ml of apatite resin. In some embodiments, the rAAV vectors present in a feedstream are isolated by elution from an apatite resin in 0-500 mM phosphate buffer (such as 0-400 mM, 0-300 mM, 0-200 mM, 0-100 mM, 0-50 mM) under conditions which retain helper virus bound to the apatite resin.

[0081] Production systems known in the art to produce rAAV vectors may include production media containing serum in the range of 0.5%-20% (v/v), or may be devoid of serum altogether. Furthermore, purification schemes described in the art may include one or more concentrations steps that may result in an increase of serum proteins and other serum components in the feedstream applied to the apatite resin. For example, the production culture supernatant as described herein which was formulated with 1% (v/v) serum was concentrated approximately twenty-fold in the TFF step, such that the feedstream loaded onto the apatite resin contained as much as 20% serum protein contaminants compared to a feedstream concentrate from a production culture formulated without serum. The inventors of the present application tested the apatite capture methods provided herein with feedstream concentrates from production cultures formulated in the presence or absence of serum. The

presence of serum proteins in the feedstream was found to have no effect on the performance of the apatite chromatography step.

Heat Inactivation of Helper Virus (Heat Kill)

[0082] If infectious adenovirus is used as a source of helper virus in the production cultures for rAAV production, an optional heat inactivation (heat kill) step can be incorporated to inactivate any residual adenoviral particles which may be present in the feedstream. The heat kill step takes advantage of one of the major differences between AAV and adenovirus: adenovirus particles are inactivated at temperatures of approximately 54-56ºC, while AAV and rAAV viral particles are stable and unaffected by those temperatures. In the present invention, the inventors have adjusted the heat inactivation step to accommodate larger scale process optimization such as the 250L scale production cultures performed herein. In particular, the apatite eluate was heat-inactivated in a sterile, single use 5 L bioprocessing bag on a temperature-controlled rocking platform set to 53°C at a rocking speed of 40 RPM, with a 12° angle for mixing (20L Wave heater pan). The apatite eluate was incubated on the platform until it reached 52°C, and then held at that temperature for an additional 10 minutes. $MgCl_2$ was added to the apatite eluate at a final concentration of 2 mM to stabilize the rAAV vector during heating. One of ordinary skill in the art can appreciate that the scale, final set point for heating, and heating time can be empirically tested to find optimal conditions to inactivate adenoviral particles while maintaining the infectivity and integrity of the rAAV particles. The heat inactivation step can be omitted for purification of rAAV particles from production cultures that utilize plasmid constructs to provide helper function.

Hydrophobic Interaction Chromatography

[0083] Hydrophobic interaction chromatography (HIC) is a technique for separating biomolecules based on differences in their surface hydrophobicity. Thus, HIC is considered an orthogonal method to the other purification steps in the AAV process. HIC chromatographic media contain hydrophobic ligands such as linear chain hydrocarbons (*e.g.*, propyl (C3), butyl (C4), hexyl (C6), or octyl (C8)) or aromatics (*e.g.*, phenyl). In pure water, the hydrophobic effect is too weak for functional interaction between the ligand and proteins, or between the proteins themselves. However, lyotropic salts enhance hydrophobic interactions, and the addition of salt drives the capture of proteins to HIC media. For this reason, HIC resins are usually loaded under high salt concentrations and eluted at lower salt concentrations. As one of ordinary skill in the art will appreciate, ammonium sulfate

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[$(NH_4)_2SO_4$] is the most commonly used salt to control capture of proteins via HIC chromatography, because of the high lyotropic ranking of both ammonium and sulfate ions in the Hofmeister series, and the high solubility of the salt. In the present invention, rAAV particles present in a feedstream were loaded onto a HIC resin by in-line mixing of a 75:25 (volume:volume) ratio of 2 M ammonium sulfate + 50 mM BisTris buffer (pH 7.0):feedstream, respectively. In-line mixing of the feedstream with the loading buffer avoids the risk of any rAAV vector precipitation by the high concentration of ammonium sulfate present in the buffer. As one of ordinary skill in the art can appreciate the concentration of salt (ammonium sulfate) can be manipulated to achieve the optimal concentration for rAAV binding. Accordingly, in some embodiments, the ammonium sulfate concentration is between 1 M and 3 M. In some preferred embodiments, the ammonium sulfate concentration in the loading buffer is 2 mM. As one of ordinary skill in the art can appreciate, in-line mixing of the ammonium sulfate and feedstream is performed for convenience and flow of the unit operation, but one could easily mix the feedstream with the appropriate concentration of loading buffer by any means known in the art and then load feedstream + loading buffer solution onto the HIC chromatographic media.

[0084] Co-solvents can also affect the hydrophobic interaction. For example, ethylene or propylene glycol can reduce the interaction between protein and the immobilized ligand and thus be useful for improving elution profiles. Accordingly, the HIC column was washed with a 75:25 (v:v) mixture of 2M ammonium sulfate + 50mM BisTris buffer (pH 7.0):50mM BisTris (pH 7.0) + 10% Propylene Glycol (v:v) buffer (EMD BioSciences), and rAAV was eluted in a low salt buffer plus propylene glycol (800 mM ammonium sulfate + 50mM BisTris buffer (pH 7.0) + 4% Propylene Glycol. Under those elution conditions, any residual helper virus and proteins present in the feedstream loaded onto the colum would remain bound to the column. The Propylene Glycol in this example was added to the buffers to sharpen the elution profile, in comparison to the broad elution profile of the buffer without Propylene Glycol, but is optional in the process.

[0085] Examples of suitable hydrophobic resins include without limitation Tosoh Butyl 650M, Tosoh SuperButyl 650C, Tosoh Phenyl 650C, and EMD Fractogel Phenyl (Tosoh Bioscience LLC, PA)

[0086] Waste from rAAV production processes requires stringent decontamination before disposal for at least two reasons: (1) the product comprises a viral vector; and (2) production cultures commonly use live adenovirus type 5 (Ad5) as a helper virus for rAAV production.

Liquid waste from chromatography operations is typically decontaminated first with bleach at point of use and then undergoes further decontamination by holding at high pH before neutralization and disposal.

[0087] The ammonium sulfate present in the HIC buffers reacts with both bleach and sodium hydroxide to release hazardous chlorine and ammonia gas respectively. Therefore, a primary consideration for process optimization of the HIC chromatography step was the development of a suitable buffer system that could be safely decontaminated by methods known in the art.

[0088] As one of ordinary skill in the art can appreciate, buffers with high salt concentrations used in hydrophobic interaction chromatography must be further screened for viscosity issues which can result in high backpressures that can either limit flow rates or cause mixing problems, thereby increasing the risk of precipitation of the product by salt crystallization in the buffers occurring at temperatures used for storage or operation. Based on the data in Table 6 below and consideration of the factors described above, the inventors selected 1 M sodium citrate (pH 7.0) as the optimal buffer for binding of rAAV vectors to the HIC chromatographic media, although citrate buffers can be used in hydrophobic interaction chromatography at concentrations ranging from 0.5 M to 2.0 M.

Buffer Exchange by Size Exclusion Chromatography (SEC)

[0089] Numerous methods are known in the art to perform the buffer exchange described herein, including TFF and dialysis. Use of size exclusion chromatography has the additional advantage of providing further protein clearance of proteins sized to pass through the pores in the resins and being relatively fast in terms of time necessary to exchange the buffers. Buffer exchange was performed at this step to ensure that the HIC eluate of the previous step was exchanged to an appropriate buffer for rAAV binding to the final anion exchange chromatography step in the process.

Adventitious Agent (Viral Clearance)

[0090] Optionally, a further step to clear trace contaminants, such as adventitious viruses which may be present in the feedstream, can be incorporated into the process, thereby yielding a commercially reasonable orthogonal process. Thus, in some embodiments, the process further includes a viral clearance filter. Examples of such filters are known in the art and include Millipore Viresolve NFR (50 nm), Pall Ultipore VF (50 nm), and Asahi 70 nm.

Anionic Exchange Chromatography

[0091] An anion exchange capture step for the rAAV vector subjected to apatite chromatography was performed as a final concentration and polish step. Suitable anion exchange chromatography media are known in the art and include without limitation, Unosphere Q (Biorad, Hercules, California), and N-charged amino or imino resins such as *e.g.*, POROS 50 PI, or any DEAE, TMAE, tertiary or quaternary amine, or PEI-based resins known in the art (U.S. Patent No. 6,989,264; N. Brument et al., *Mol. Therapy* 6(5):678-686 (2002); G. Gao et al., *Hum. Gene Therapy* 11:2079-2091 (2000)). One of ordinary skill in the art can appreciate that wash buffers of suitable ionic strength can be identified such that the rAAV remains bound to the resin while other in-process impurities including without limitation glucans which may be introduced by leaching from various filters utilized in the purification steps are stripped away. In some embodiments, the wash buffer is 60 mM NaCl and the rAAV vector is eluted from the column with 130 mM NaCl, such that any residual trace in-process impurities present, such as serum albumin or helper virus, are retained on the column.

EXAMPLES

Example 1: Harvesting rAAV-1 from culture medium-clarification & Benzonase® digestion

[0092] Spent rAAV-1 production medium (supernatant) from a 250 L rAAV-1 viral production culture produced by any method known in the art containing the rAAV-1 vector was clarified to remove any cells contained in the supernatant. The supernatant was passed through a series of filters connected in series, including: (1) a Millipore Millistak+® HC Pod Filter, Grade D0HC (Millipore Corp., Bedford, MA)(4 times); (2) a Millipore Millistak+® HC Pod Filter, Grade A1HC; and (3) an Opticap® XL10 Millipore Express SHC Hydrophilic Membrane 0.2 μm Filter at a rate of 5 liters per minute (LPM) which was reduced stepwise to 4 LPM.

[0093] All filters were prewashed in reverse osmosis/de-ionized ("RO/DI") water per manufacturer's specifications. The flow-through was collected into a bioprocess bag for Benzonase® digestion. A final concentration of 2 units/ml of Benzonase® (EM Industries catalog number 1.01695.0002) was dissolved in the rAAV-1 production medium and added to the clarified viral supernatant to achieve a final concentration of 2.5 units/ml. The supernatant plus Benzonase® was incubated at ambient temperature with constant

recirculation at 4 LPM to allow for DNA digestion. Data from the Benzonase® digestion is shown in Figure 1, which demonstrates that no high molecular weight DNA was present following Benzonase® digestion.

Example 2: Removal of production contaminants via anionic exchange

[0094] The rAAV-1 clarified and Benzonase®-digested supernatant from Example 1 was passed over a series of two-inch by twenty-two-inch Pall Mustang® Q ("MQ") filters connected in series (Pall Corp., catalog number NP6MSTGQP1). Prior to loading of the rAAV-1 viral, supernatant the filters were sanitized with 15 L of 0.5 M NaOH at 0.5 LPM with a holding time of 15 minutes, charged by rinsing with 15 L TMEG + 2M NaCl (TMEG: 0.05 M Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 1 mM Na_2EDTA, 10% (v/v) glycerol) at a rate of 6 LPM, and equilibrated with 15 L of vector production medium at 6 LPM. The supernatant was then pumped at a rate of approximately 6 LPM through the series of filters and collected into a bioprocessing bag. At the ionic strength of the production medium, the anionic exchange MQ filter was demonstrated to clear helper virus and residual DNA, among other impurities, from the rAAV-1 supernatant by binding of the contaminants to the charged membrane. At the ionic strength of the production culture, however, the rAAV-1 vector present in the supernatant flowed through the anionic exchange membrane. During process optimization it was determined experimentally that using a single MQ filter resulted in a breakthrough of contaminants in the process, including the Ad5 helper virus. Consequently a second filter was added in series or in tandem in the process.

Example 3: Concentration of rAAV-1 vector supernatant

[0095] The complete rAAV-1 vector production supernatant processed in Examples 1 and 2 was concentrated approximately 20-fold via tangential flow filtration ("TFF") from an initial volume of approximately 250 L to a volume of approximately 12.5 L. Tangential flow polyethersulphone filter cartridges with a 100 kD molecular weight cut off, C screen and a 5 m^2 total surface area (Millipore Pellicon® 2 Biomax, Catalog No. P2B100C05) were flushed with 50 L of RO/DI water, sanitized with 15 L of 0.5 M NaOH with a 15 minute hold step, flushed again with 100 L of WIFI (HyPure™ WFI purified water; HyClone, Logan, UT), flushed with 15 L TMEG + 2M NaCl, and finally equilibrated with 15 L of rAAV-1 production medium. The supernatant was passed through the TFF cartridge at a flow rate of approximately 3 LPM with a recirculation rate of 16 LPM. The TFF material retained on the

filter ("retentate") was concentrated to approximately 10.5 L and was transferred to a reservoir. The filter was flushed with approximately 2 L of production medium. The wash and concentrate were then pooled to yield a final volume of approximately 12.5 L.

[0096] Concentration of rAAV-1 to a volume of 12.5 L by TFF also concentrated the remaining rAAV-1 production contaminants in the solution by approximately 20-fold. Thus, a manufacturing hold step was introduced following the TFF step, during which the TFF concentrate was filtered though a 4-inch Opticap 0.22 μM filter membrane (Millipore Opticap® Catalog No. KVSC04HB3). This extra filtration step enables the rAAV-1-containing TFF concentrate to proceed to the next step in the process without requiring diafiltration and buffer exchange. The post-TFF material may be stored at 2-8ºC for any period of time, including as little as 24 hours to as much as 3 months or more, before further processing with no loss in stability as measured by vector yield or infectivity assessed by assays known in the art. Alternatively, TFF performed as described herein could be used at any step in the purification process to concentrate or buffer exchange the rAAV-1 vector .

Example 4: Resin screening for rAAV-1 vector versus process impurity binding

[0097] Commercial FDA-approved processes for purification of proteins and other biologic products relies upon commercial-scale incorporating orthogonal processes. Orthogonal processes are processes which have more than one step or process for removal of in-process impurities, including both capture and flow-through steps for the final product such as, for example, an rAAV-1 vector. rAAV vectors (specifically rAAV-2) have been demonstrated in the art to bind anionic resins. rAAV vectors such as rAAV-1, -5, and -8 have been demonstrated to bind much less tightly than rAAV-2 to anionic exchangers in the presence of production components such as serum albumin, helper virus components, production media components and host cell DNA resulting in a less efficient and lower quality purification scheme.

[0098] Previous purification strategies described in the art for lower affinity anionic binders such as AAV-1 include an iodixinol step gradient which reduces the relative concentration of the production components in order to achieve a tighter binding of the rAAV vector to anionic exchangers. Iodixinol step gradients are not readily scalable to commercial scale processes such as those described herein. Therefore, in order to optimize rAAV vector purification for low affinity anionic binders such as rAAV-1 without the need to perform ultracentrifugation and step gradients, a number of resins were screened for their ability to bind rAAV vectors or exclude the rAAV vectors in the flow-through as compared to the

ability to bind or flow-through commonly observed process impurities including host cell DNA, helper virus, serum albumin, serum proteins (if serum is included the production medium), and other low molecular weight proteins found in the production cultures in order to develop a commercially scalable, orthogonal, and efficient rAAV purification process.

[0099] Resin screening was performed using a 1 ml (5 cm bed height) column with linear flow rates recommended by the vendor for each resin at severely underloaded capacities relative to the manufacturer's recommendations. Spectrophotometric tracings of ultraviolet absorbance at 280 nanometers (A_{280}) were collected for binding to and elution from each resin. Peaks were analyzed by the appropriate assay for both rAAV-1 vector and representative process impurities. The data presented in Figure 2 represents a typical spectrophotometric tracing for a typical resin in the list. Table 2 lists some of the resins screened as well as the relative binding affinities of the resin for the rAAV-1 vector and various process impurities.

Table 2: Screening of resins for binding of rAAV-1 compared to binding of process contaminants

Resin	Resin Type	rAAV-1	Helper Virus	Host Cell DNA	Serum Albumin	Serum Proteins
UnoS pH 5.5	Cation Exchange	+	0	–	+	+
UnoS pH 7.0	Cation Exchange	–	–	–	–	–
UnoQ pH 8.5	Anion Exchange	+	++	++++	++	++
UnoQ pH 7.0	Anion Exchange	+	+++	++++	++	++
Fractogel EMD SO_3	Cation Exchange	+	++	–	0	0
CHT	Apatite	+	0	++	+	+
CFT	Apatite	+	0	++	+	+
HIC Phenyl	Hydrophobic Exchange	–	–	–	0	0

Resin	Resin Type	rAAV-1	Helper Virus	Host Cell DNA	Serum Albumin	Serum Proteins
HIC Butyl	Hydrophobic Exchange	+	++	–	0	0
HIC Hexyl	Hydrophobic Exchange	+	++	–	0	0
HIC PPG	Hydrophobic Exchange	–	–	–	0	0
Source S	Ion Exchange	+	0	0	++	0
Source Q	Ion Exchange	+	0	0	++	0
TMAE	Ion Exchange	+	0	0	++	0
IMAC $FeCl_3$		0	0	–	+	+
Superdex 200	Gel Filtration	void	void	chase	chase	chase
HW55	Gel Filtration	void	void	chase	chase	chase
HW65	Gel Filtration	void	void	chase	chase	chase
HW75	Gel Filtration	void	void	chase	chase	chase

Key: (–) = present in flow-through (no binding); + = weakly bound (eluted very early in the gradient); ++ to ++++ = stronger binding (eluted further along the gradient).

Example 5: Development of apatite chromatography in the presence of polyethylene glycol ("PEG") for capture of rAAV-1

[0100] Based on the results of the resin screening performed in Example 4, an apatite resin or ceramic apatite resin was chosen as one of the capture resins for rAAV-1. Initial experiments were performed using CFT II resins, but for later purification the resin was changed to CHT I, as discussed in detail below. Data indicated that both chromatographic resins performed equivalently. Experiments were performed to further increase the rAAV-1 binding capacity of the apatite resin and improve the ability of the resin to discriminate between rAAV-1 particles and other in-process impurities. Two key improvements to the function of the apatite resins were further developed as described herein: 1) the addition of PEG; and 2) development of the loading buffer conditions.

[0101] Based on the variable breakthrough of the apatite column due to capacity issues at commercially reasonable column sizes, PEG was mixed with the TFF concentrate (*see* Example 3 above) before loading on the apatite resin in order to increase binding of the rAAV-1 vector relative to other in-process impurities such as serum albumin, helper virus and other protein impurities which out-competed the rAAV-1 vector for binding to the column in Example 4.

[0102] Apatite chromatography in the presence of PEG represents an efficient capture or binding strategy for purification of rAAV-1 vectors, although many in-process impurities were also retained by the apatite resin at pH 7.0.

[0103] Experiments were performed to determine if modifying the buffering conditions could improve the resolution of rAAV-1 from other in-process impurities. Small scale experiments were performed using an AKTAexplorer FPLC System (GE Healthcare, Piscataway, NJ) equipped with 1.2 mL Tricorn 5 columns (GE Healthcare) packed at a 6 cm bed height with CFT resin, run at a flow rate of 150 cm/hr. Those columns were evaluated for rAAV-1 vector capture versus binding of bovine serum albumen ("BSA"), a model small molecule in-process impurity, in various buffer systems in the presence or absence of 5% PEG6000.

[0104] rAAV-1 or BSA was injected in small volumes (<5% of total volume) on the CFT column in the buffer system to be tested, either in the presence or absence of 5% (w/v) PEG6000. Small volumes were used in order to obviate the need for buffer exchange of the samples. Products were eluted along a 500mM PO_4 gradient. 50 mM 2-(N-morpholino) ethanesulfonic acid ("MES") was used to buffer the system at pH=6.50, and 20 mM borate was used to buffer the system at pH 9.0.

[0105] The data presented in Table 3 demonstrate that rAAV-1 vector binding to the apatite resin was essentially the same at pH 6.5 or pH 9.0 in the presence of 5% (w/v) PEG6000, while binding of BSA, the model small molecule in-process impurity, was dramatically reduced at pH 9.0 in the presence or absence of 5% (w/v) PEG6000, as indicated by the spectrophotmetric tracings (data not shown). Further analysis of the reduced capacity of BSA to bind to the apatite column at basic buffer loading conditions (*i.e.*, pH=9.0) by enzyme-linked immunosorbent assay ("ELISA") demonstrated that most BSA (~78%) was present in the flow-through at pH 9.0 buffer conditions, while additional levels of clearance or reduction in BSA binding could be achieved during subsequent wash steps (~19%), leaving only ~0.1% of the BSA loaded onto the column actually bound to the apatite resin

and co-eluting with the rAAV-1 vector. rAAV-1 particles were stable at pH=9.0, as indicated by no loss of infectivity or decrease in the number of DNase-resistant particles ("DRP") eluted.

Table 3: Relative strength of binding of rAAV-1 and BSA to apatite resin at pH=6.5 or pH=9.0, with or without 5% (w/v) PEG6000

Binding conditions	BSA	5% (w/v) PEG6000
pH=6.50	++	+
pH=6.50 + 5% (w/v) PEG6000	++	++++
pH=9.0	+	+
pH=9.00 + 5% (w/v) PEG6000	+	++++

Key: + = weak binding; ++ = medium binding; ++++ = strong binding.

Example 6: Effect of serum in the rAAV-1 harvest culture on rAAV-1 capture via apatite chromatography in the presence of PEG

[0106] rAAV-1 vector production cultures or rAAV-1-containing feedstreams to be purified by the methods described herein may contain serum and serum proteins if the production cultures were grown in medium containing serum. While rAAV-1 vector production that uses very low concentrations of serum (*i.e.*, 1% or less)(*see, e.g.*, U.S. Pat. No. 6,995,006) has been described, concentration of the production culture or feedstream as described in Example 3 can produce a feedstream that effectively contains 20% serum and serum proteins as a result of the 20-fold concentration of the production culture harvest. In order to evaluate the effect of serum components on performance of the apatite chromatography, experiments were performed on production cultures produced in the presence or absence of serum, in the presence or absence of PEG6000.

[0107] Two model rAAV-1 production cultures were used to assess capacity of the apatite resin (CFT type I) by traditional breakthrough analysis in a total of four column loading experiments. In one experiment, the production cultures contained serum; and in the second experiment, the production culture did not contain serum. Both feedstreams were tested in the presence of 5% (w/v) PEG6000 or in the absence of PEG. Feedstreams were

representative of the harvest process, and were clarified culture supernatants that had been passed over an anion-exchange filter and concentrated 20-fold by tangential flow filtration as described herein. CFT type I columns were loaded by 1:1 online mixing of the feedstream with a borate buffer at pH=9.0. The buffer contained either 0% or 10% (w/v) PEG6000 (to achieve a final concentration of 5% (w/v) PEG6000). As columns were loaded, the flow-through was collected in a series of fractions which were analyzed for product by DRP-PCR. Functional capacity was defined as the point at which the product concentration at the outflow of the column just reached 1% of the concentration entering the column, after accounting for the online dilution. For column loads containing PEG, the remainder of the chromatography process was then run to assess vector recovery in the elution fractions.

[0108] The data presented in Figure 3 demonstrates that the addition of PEG6000 increases the capacity of the apatite resin for rAAV-1 vector binding regardless of whether serum was present in the production culture. Without wishing to be bound by theory, The post-TFF supernatant in the presence of PEG preferentially binds rAAV-1 to the apatite resin over other in-process impurities via an anionic interaction to the phosphate moieties which can be outcompeted by the presence of phosphate in the elution buffer. In addition, rAAV-1 binding to the apatite resin is further discriminated from in-process impurities via the metal interaction which can be outcompeted by the presence of salt. The binding of rAAV-1 to the phosphate moieties is not driven primarily by hydrophobicity, as the capture and elution buffers are formulated to be primarily ionic in nature (*i.e.*, the elution buffer contains 50 mM phosphate, compared to elution buffers containing 150 mM phosphate or higher commonly used in eluting compositions bound by primarily hydrophobic interactions). Under these high salt, low phosphate elution conditions, residual helper virus, host cell DNA, and other low molecular weight proteins contained in the supernatant of the production cultures would, if present, be retained on the resin. Surprisingly, the data demonstrates that in the presence of PEG6000, rAAV-1 vectors produced in either serum-containing or serum-free media demonstrate a binding capacity for the apatite resins of at least 1.2×10^{12} DRP/mL (a 1mL load) to greater than 1.5×10^{14} DRP/mL of resin (150mL). In the absence of 5% (w/v) PEG6000, the binding capacity of the apatite resin for the TFF harvest was less than 2.4×10^{12} DRP/mL for vectors produced in serum containing media and 7.2×10^{12} DRP/ml for vectors produced in serum-free media, as no rAAV-1 vector was recovered in the CFT eluate.

Example 7: Purification of rAAV-1 via apatite chromatography

[0109] The CHT Type I column was packed with 2 M NaCl and sanitized with 1 M NaOH. Before loading, the column was equilibrated with 6 column volumes ("CV") of 20 mM borate (pH = 9) + 5% (w/v) PEG6000. The TFF feedstream concentrate was loaded via a 3mm BioProcess Skid (GE Healthcare) onto a 923 ml (14 cm diameter × 6 cm bed height) CHT column prepared as described previously at a flow rate of 96 cm/hr. The TFF feedstream concentrate was mixed in-line with an equal volume of a 40 mM borate (pH = 9) + 10% (w/v) PEG6000 buffer to yield a final concentration of 20 mM borate (pH = 9) + 5% (w/v) PEG6000.

[0110] A series of 4 sequential washes were performed to remove in-process impurities while retaining the rAAV-1 vector on the column. Wash 1 ("the chase") was performed by in-line mixing of 5 CV of a 50:50 (volume:volume) 20 mM borate (pH = 9.0) + 5% (w/v) PEG6000:40 mM borate (pH = 9.0) + 10% (w/v) PEG6000 to chase all loading lines with PEG6000. Further, this step was found to preferentially increase the binding affinity of the rAAV-1 vectors. Wash 2 was performed with 15 CV of 150 mM potassium phosphate + 20 mM borate (pH = 9) + 5% (w/v) PEG6000 to remove the majority of the serum albumin and other low molecular weight protein in-process impurities while retaining rAAV-1 on the column. Wash 3 ("WII" in Figure 5)was performed with 15 CV of 20 mM borate (pH = 9) + 5% (w/v) PEG6000 to remove any residual phosphate so that the rAAV-1 remained bound to the column once the PEG6000 was removed. Wash 4 ("WIII" in Figure 5) was performed with 5 CV of 20mM HEPES (pH = 7.0) + 150mM NaCl buffer to remove the PEG6000 and to adjust the salt concentration, thereby allowing discrimination between rAAV-1 and any residual helper virus or other in-process impurities, such as protein contaminants, which may remain bound to the column.

[0111] The rAAV-1 vector was eluted from the column by 6 CV of a 50 mM potassium phosphate + 20 mM HEPES (pH = 7.0) + 150 mM NaCl buffer. Figure 4 shows a typical spectrophotometric trace of UV absorbance at 280 nm (A_{280}) and conductivity for the CHT I chromatographic procedure. Figure 5 shows the relative purity of rAAV vectors eluted from the apatite resin.

Glucan clearance by apatite chromatography

[0112] Glucans are carbohydrates similar to cellulose which leach into the process from the cellulose-based depth filter used to harvest rAAV-1 particles from production cultures.

Glucans at concentrations above ~1 ng/mL can interfere with standard *Limulus* amoebocyte lysate ("LAL") tests for bacterial endotoxin contamination. As demonstrated in Table 4 below, the apatite CHT Type I column cleared ~2.5 logs of glucans from the production culture. Under the buffer conditions described herein, the vast majority of the glucans were present in the flow-through and did not bind to the column.

Table 4: Glucan clearance in the process

Processing step	**Glucan concentration (ng/mL)**	**Total amount per lot (ng)** (250L scale)
Clarification	10.4	2,600,000
TFF	136	1,541,016
CHT elution	1.9	4,769
Post HIC and SEC	0.2	662
Final Anion Exchange Eluate	0.1	71

Samples were assayed for glucan using an LAL-based kinetic chromogenic assay specific for glucans (Glucatell®, Cape Cod, MA).

Ad5 helper virus clearance by apatite chromatography

[0113] To confirm that CHT chromatography cleared Ad5 from the feedstream, a preliminary spike-in study was performed using feedstream from the final upstream process. Ad5 spike levels were set based on data obtained from the phase I viral clearance study with CFT II resins, and three different load ratios of feedstream were used: 6.6 mL; 13.5 mL; and 33 mL of post-TFF feedstream per mL CHT resin.

[0114] The data presented in Table 5, Ad5 clearance by CHT was comparable to the 4 LRV clearance demonstrated approximately 4 logs of Ad5 viral clearance and appeared to be independent of volume of feedstream loaded, within the 5-fold range assessed. The low Ad5 recovery is consistent with previous data indicating that under the buffer conditions utilized the Ad5 binds tighter than rAAV-1 to the apatite resin.

Table 5: Total infectious units of Ad5 in CHT column fractions

Load ratio / Fraction	6.6 mL	13.5 mL	33 mL
Load	9.0×10^{8}	1.3×10^{9}	9.0×10^{8}
Flowthrough + chase	$<2.5\times10^{5}$	$<3.6\times10^{5}$	$<6.9\times10^{5}$
PO_4 Wash	2.9×10^{6}	2.7×10^{6}	$<2.6\times10^{6}$
Washes II & III	3.6×10^{6}	2.2×10^{6}	2.8×10^{6}
Elution	$<6.9\times10^{4}$	$<6.9\times10^{4}$	$<3.5\times10^{5}$
Log reduction value (LRV)	**>4.1**	**>4.3**	**>3.4**

[0115] A total of 8×10^{9} infectious particles of Ad5 (*i.e.*, total particles with a P:I of ~10) were spiked into different volumes of post-TFF feedstream to run on 1.2 mL CHT columns. Columns were run at 100 cm/hr and fractions were collected to assay for vector by DRP and Ad5 by infectious titer assay. A spike-controlled version of the Ad5 infectivity assay was used since high concentrations of both the load and CHT elution samples are known to interfere in the cell-based assay. Ad5 clearance was determined as the Log Reduction Value ("LRV"), calculated as the logarithm of the total amount of Ad5 loaded divided by the total amount of Ad5 recovered in the elution fraction (Log Reduction Value).

Example 8: Heat inactivation of residual helper virus

[0116] A heat inactivation step was performed in order to inactivate and remove any residual helper virus present in the CHT I eluate. For smaller scale experiments, the CHT I eluate was divided between two 1 L PETG (Nalgene®) bottles and $MgCl_2$ added to a final concentration of 2 mM in order to increase the stability of the rAAV-1 vector. The bottles were incubated in a 53.5°C water bath with mixing until the temperature in the bottle reached approximately 52°C. The bottles were then cooled by transfer to an ambient temperature water bath and mixed until the temperature in the bottle was no greater than 5°C above ambient temperature. The heat-killed mixture was filtered through a 4-inch Opticap® 0.22 μM filter membrane (Millipore Opticap® catalog number KVSC04HB3). Alternatively, for larger scale experiments, the CHT I eluate was heat inactivated in a sterile, single use bioprocessing bag (Custom Hyclone 5 L bag, CX5-14 film) on a temperature controlled rocking platform with a temperature set-point of 53°C at a rocking speed of 40 RPM and a mixing angle of 12° (20 L wave heater pan). The CHT I eluate was incubated on the

platform until the temperature reached 52°C and then held for an additional 10 minutes. To stabilize the rAAV-1 during heating, $MgCl_2$was added to 2 mM final concentration. After heating, the product was filtered through a 0.2 μm filter and held overnight at ambient temperature to minimize possible temperature effects on the subsequent hydrophobic interaction column.

Example 9: rAAV-1 Capture via Hydrophobic Interaction Chromatography ("HIC")

[0117] HIC is a technique for separating biomolecules based on differences in their surface hydrophobicity. As such, HIC is considered an orthogonal method to the other purification steps in the rAAV-1 process. HIC media contain hydrophobic ligands such as linear chain hydrocarbons (*e.g.*, propyl (C3), butyl (C4), hexyl (C6), or octyl (C8)) or aromatic hydrocarbons (*e.g.*, phenyl). In pure water, the hydrophobic effect is too weak for functional interaction between the ligand and proteins, or between the proteins themselves. However, lyotropic salts enhance hydrophobic interactions, and adding such salts drives adsorption of proteins to HIC media. For this reason, HIC resins are usually loaded under high salt concentrations and eluted at lower salt concentrations.

HIC chromatography with ammonium sulfate buffers

[0118] Briefly, a 170 ml (6 cm diameter × 6 cm bed height) HIC butyl column (Toyopearl® Butyl 650M; Tosoh Biosciences, Montgomeryville, PA; Catalog number 14702) was sanitized with several column volumes of 0.5 M NaOH and equilibrated with a 75:25 (volume:volume) mixture of 2 M ammonium sulfate + 50 mM Bis Tris (pH = 7.0):50 mM Bis Tris (pH = 7.0). The heat killed rAAV-1 vector apatite eluate was loaded at a rate of 3.3 L/hr with in-line mixing at a 75:25 (volume:volume) ratio of 2 M ammonium sulfate + 50 mM Bis Tris (pH = 7.0):rAAV-1 apatite eluate. In-line mixing avoids the risk of any rAAV-1 vector precipitation by the ammonium sulfate present in the buffer. The column was washed with one or more column volumes of a 75:25 (volume:volume) of a 2 M ammonium sulfate + 50 mM Bis Tris pH = 7.0 buffer:50 mM Bis Tris pH = 7.0 + 10% Propylene Glycol (volume:volume) (EMD Biosciences) buffer. The propylene glycol in this example was added to the buffers to sharpen the elution profile, compared to the broad elution profile of the buffer without propylene glycol, although it is optional in the process. The rAAV-1 vector was eluted from the column with 800 mM ammonium sulfate + 50 mM Bis Tris (pH = 7.0) buffer + 4% propylene glycol. At the elution conditions utilized, any residual helper virus and proteins present in the load would remain bound to the column.

[0119] Waste from rAAV-1 production processes requires stringent decontamination before disposal, due to both the product being a viral vector and the use of live adenovirus type 5 (Ad5) as a helper virus for production. Liquid waste from chromatography operations is typically decontaminated first with bleach at point of use and then further decontaminated by holding at high pH before neutralization and disposal. Ammonium sulfate present in the HIC buffers reacts with both bleach and sodium hydroxide to release hazardous chlorine and ammonia gas respectively. Therefore, a primary consideration for process optimization of the HIC chromatography step was the development of a suitable buffer system that could be safely decontaminated by methods known in the art.

Screening for Suitable Buffers for rAAV-1 binding to the HIC column

[0120] rAAV-1 vector was loaded onto columns in a variety of different buffer conditions and relative binding efficiency was determined by measuring the amount of rAAV-1 vector present in the flow-through fraction (Table 6). Buffers evaluated included both high concentration lyotropic salts traditionally utilized with HIC chromatographic processes and several low pH buffers where a mixed-mode interaction (HIC/cation exchange) could potentially occur. Both the Tosoh Butyl 650M and EMD Phenyl resins bound vector in several of the alternative buffers.

[0121] Buffers with high salt concentrations used in hydrophobic interaction chromatography must be further screened for viscosity issues which can result in high backpressures that can either limit flow rates or cause mixing problems and the risk of precipitation of the product due to salt crystallization at storage or operating temperatures of the buffers. Based on the data in Table 6 below, and after considering the factors described above, 1 M sodium citrate, pH = 7.0 was chosen as the optimal buffer for binding of rAAV-1 vectors to the HIC chromatographic media.

Table 6: Screening for AAV1 binding in alternative buffers

Buffer System Tested	Butyl 650M (% in flow-through)	EMD-Phenyl (% in flow-through)
1.1 M Sodium Sulfate (pH = 7)	**0%**	**0%**
1 M Sodium Citrate (pH = 7)	**0%**	**0%**
1.3 M Potassium Phosphate (pH = 7)	3%	3%
2.9 M NaCl, 50 mM Sodium Citrate (pH = 4)	**0%**	28%
1 M Glycine, 50 mM Sodium Citrate (pH = 4)	4%	1%
50 mM Potassium Phosphate (pH = 4.5)	3%	NT
50 mM Sodium Citrate (pH 4)	4%	NT
2.9 M NaCl, 50 mM Potassium Phosphate (pH 4.5)	17%	NT

[0122] Experiments were performed at ambient temperature on Tricorn 5/50 columns (6 cm bed height, 1.2 mL column volume) using purified rAAV-1 vector. Columns were equilibrated with the buffers listed and ~2×10^{11} DRP or rAAV-1 were loaded on the column. rAAV-1 was eluted over a 20 CV linear gradient from 145 mM Bis Tris (pH = 7.0), 10% (v/v) propylene glycol. The flow-through was collected and assayed by DRP analysis for the fraction of rAAV-1 applied to the column that flowed through or did not bind.

[0123] Further characterization was performed on the clearance of in-process impurities on the HIC column in the various buffers demonstrating good binding of rAAV-1 in the previous experiment. The data in Table 7 below for model contaminant binding demonstrates that both adenovirus and DNA if present in the feedstream are effectively discriminated by the HIC chromatographic step.

Table 7: Relative binding of rAAV-1 versus model in-process impurities in different HIC buffers

Buffer System	rAAV-1	Ad5	DNA
0.1 M sodium sulfate + bis tris buffer, pH = 7.0	+	++	–
0.8 M sodium sulfate, bis tris buffer, pH = 7.0	+	++	0

1 M sodium citrate, pH = 7.0	+	++	0
2.9 M NaCl (50 mM sodium citrate to buffer at pH = 4.0)	–	–	0

Key: "0" = no binding material present in flow-through; "–" = very weak binder; "+" = strong binder; "++" = stronger binder.

[0124] Experiments were performed at ambient temperature on Tricorn 5/50 columns (6 cm bed height, 1.2 mL column volume). Columns were equilibrated with the buffers listed and the indicated samples were loaded on the column. Each sample was eluted over a 20 CV linear gradient. Samples were collected and assayed for the relevant load material.

HIC chromatography with sodium citrate buffers

[0125] The heat-killed rAAV-1 vector apatite eluate was subsequently loaded onto a HIC butyl column in order to further reduce any residual process impurities and as a concentration and desalting step. A 373 ml (6 cm diameter × 8.9 cm bed height) HIC butyl column (Tosoh Biosciences Toyopearl® Butyl 650M catalog number 14702) was sanitized with several column volumes of 0.5 M NaOH and equilibrated with 5 CV of a 75:25 (volume:volume) mixture of 1 M Citrate + 20 mM sodium phosphate:20mM sodium phosphate. The heat-killed rAAV-1 vector CHT I eluate was loaded at a rate of 106 cm/hr with in-line mixing at a 75:25 (volume:volume) ratio of 1 M citrate + 20mM sodium phosphate:CHT I eluate. In-line mixing avoids the risk of any rAAV-1 vector precipitation. The column was washed with 5 CV of a 75:25 (volume:volume) mixture of 1 M citrate + 20mM sodium phosphate:20mM sodium phosphate buffer. The rAAV-1 vector was eluted from the column with 6 CV of 0.35 M citrate + 20 mM sodium phosphate. The column was then washed with 3.5 CV of 20 mM sodium phosphate buffer. This low salt wash (20mM sodium) elutes a fraction of rAAV-1 vector particles that are hydrophobically distinct in their elution profile from the rAAV-1 vector particles that elute in the higher salt elution buffer. In fact, if the low salt eluted fraction was isolated and reapplied to the HIC column under the conditions described, that population of vector still eluted only in the low salt fraction, indicating that the fraction was not the result of a breakthrough in capacity of the column. Infectivity analysis suggests that this fraction of rAAV-1 likely represents a population comprising empty capsids, partially

denatured capsids, less infectious capsid material, and partially full capsids. Therefore, this observation may lead to improvements in the separation of rAAV-1 particles that are less infectious and therefore less desirable as product material. At the elution conditions utilized any residual helper virus and proteins present in the load would remain bound to the column and thus should be present in the low salt strip.

Example 10: Buffer exchange by size exclusion chromatography ("SEC")

[0126] Buffer exchange by size exclusion chromatography provides additional protein clearance of proteins sized to pass through the pores in the resins, and is relatively fast in terms of time necessary to exchange the buffers. The buffer exchange performed at this step was to ensure that the HIC eluate of the previous step was exchanged to an appropriate buffer for rAAV-1 binding to the final anion exchange chromatography step in the process. A 3.2 L (14 cm diameter × 21 cm bed height) Amersham Superdex® 200 prep grade resin (Amersham/GE Healthcare, Piscataway, NJ; Catalog number 17-1043-04) was packed and prepared by sanitizing with 2 M NaCl + 1 M NaOH and equilibrated with 2.8 CV of 20 mM NaCl + 20 mM Tris (pH = 8.0). The HIC elution was sub-divided to process over the SEC in three sequential cycles of approximately 400ml each, loading no more than 12.5% of the SEC column volume for each cycle. The product peaks (contained in the void volumes) from the three SEC cycles were collected in a single bioprocessing bag. The HIC eluate was loaded onto the column at a flow rate of 49 cm/hr. The column was chased and flushed with 1.4 CV of 20 mM NaCl + 20mM Tris (pH = 8.0) and the rAAV-1 vector present in the HIC eluate was present in the void volume of the column. Following the collection of the void volume as described, the second and third fractions were loaded and collected on the same column sequentially as described previously for the first fraction.

Example 11: Adventitious agent (viral clearance)

[0127] As an optional process to clear adventitious viruses which may be present as trace contaminants and thus yield a commercially reasonable orthagonal process, a viral clearance filter was introduced into the process. Examples of such filters are known in the art and include Millipore Viresolve® NFR (50 nm), Pall Ultipore® VF (50 nm), and Asahi 70 nm. A Millipore Viresolve® NFR (Millipore 4" Virosolve NFR filter Catalog number KZRV 04T C3) viral clearance filter was prepared according to manufacturers instructions, flushed with 20 mM NaCl + 20 mM Tris (pH = 8.0) and the SEC elution was filtered through the

membrane. The filter was flushed with several volumes of 20 mM NaCl + 20 mM Tris-HCl (pH 8.0) and pooled with the filtered SEC eluate.

Example 12: Anionic Exchange Chromatography

[0128] A second anion exchange capture step for the rAAV-1 vector was performed as a final concentration and polish step on a Unosphere® Q resin (Biorad, Hercules, CA). A 373 ml (8.9 cm diameter × 6 cm bed height) column was sanitized with several column volumes of 0.5 M NaOH and equilibrated with 7 CV of 20 mM NaCl + 20 mM Tris (pH = 8.0) buffer. The SEC void volume fraction or optionally the viral filtered eluate was loaded at a rate of 309 cm/hr. The column was washed 10 CV of 60 mM NaCl. The ionic strength of the wash solution was chosen to retain rAAV-1 bound to the resin while stripping away any other in-process impurities, such as glucans which may be introduced by leaching from various filters utilized in the purification steps. The rAAV-1 vector was eluted from the column with 6 CV of a 130 mM NaCl. The ionic strength of the 130 mM NaCl salt elution will strip rAAV-1 from the column while any residual trace in-process impurities, such as serum albumin or helper virus would remain bound.

[0129] Figure 6 compares the degree of purification across the various process steps by SDS-PAGE. In-process samples from a representative production culture harvest were run on a denaturing/reducing 10% polyacrylamide gel and stained with Sypro Orange. All post-harvest samples were loaded at 1×10^{10} DRP / lane. The two upstream samples before the TFF concentration step (initial clarification step and anion exchange ("AEX") flow-through) could only be loaded at 1×10^{9} DRP/lane due to volume constraints on the gel. Beta-galactosidase (B-Gal) was loaded at 50 ng/lane to assess sensitivity and consistency of staining across the gel. The three AAV1 capsid proteins (VP1, 2, and 3) are indicated.

Example 13: Percent Recovery of rAAV during Purification

[0130] The data presented in Figure 7 shows the percentage recovery of infectious rAAV particles after each process step of the purification scheme from a representative production culture of an rAAV-1 vector. The percentage recovery was calculated based on the total DRPs of the rAAV-1 vector recovered from each process step divided by the total number of DRPs loaded or subjected to that purification step. The data demonstrates that at each step in the purification process, recoveries of approximately 60% or greater were achieved. In numerous experiments, the range of recovery from each process step was at least 60% to

90%. Particularly noteworthy, the range of recovery at the capture step (*i.e.*, the apatite chromatography step) in individual experiments ranged from 57% to greater than 90%. Furthermore, the range of recovery at the HIC step ranged from 60% to 80%.

[0131] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is apparent to those skilled in the art that certain minor changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention.

Novartis Exhibit 1002, Page 432 of 591

CLAIMS

1. A method for isolating a population of recombinant adeno-associated virus (rAAV) particles from in-process impurities in a feedstream, comprising the steps of:

(a) contacting a feedstream containing the rAAV particles with an apatite chromatography medium in the presence of polyethylene glycol (PEG), wherein the rAAV particles bind to the apatite chromatography medium; and

(b) eluting the rAAV particles bound to the apatite chromatography medium with an elution buffer containing less than 3% (w/v) PEG.

2. The method of claim 1, wherein the apatite chromatography medium is ceramic hydroxyapatite (CHT).

3. The method of claim 1, wherein the apatite chromatography medium is ceramic fluoroapatite (CFT).

4. The method of claim 1, wherein the specific binding of the apatite chromatography medium to the rAAV particles is between 10^{14} and 10^{16} DNase-resistant particles per milliliter (DRP/mL).

5. The method of claim 1, further comprising a step of binding the rAAV particles in the feedstream eluted from the apatite chromatography medium to an anionic chromatography medium.

6. The method of claim 1, wherein the feedstream containing the rAAV particles in step (a) is contacted with an apatite chromatography medium in the presence of polyethylene glycol (PEG) and a basic buffer.

7. The method of claim 6, wherein the basic buffer is between pH 7.6 and 10.

8. The method of claim 6, wherein the basic buffer is between pH 8.0 and 10.0.

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9. The method of claim 6, wherein the basic buffer is between pH 9.0 and 10.0.

10. The method of claim 6, wherein the basic buffer comprises borate.

11. The method of claim 1, wherein the PEG has an average molecular weight between about 5,000 (PEG5000) grams per mole and about 15,000 (PEG15000) grams per mole.

12. The method of claim 11, wherein the PEG has an average molecular weight of about 6,000 (PEG6000) grams per mole.

13. The method of claim 1, wherein the feedstream containing the rAAV particles in step (a) is contacted with the apatite chromatography medium in the presence of between about 3% (w/v) and about 10% (w/v) PEG.

14. The method of claim 13, wherein the feedstream is contacted with the apatite chromatography medium in the presence of about 5% (w/v) PEG6000.

15. The method of claim 13, wherein the feedstream is contacted with the apatite chromatography medium in the presence of about 10% (w/v) PEG6000.

16. The method of claim 1, further comprising a step of washing the apatite chromatography medium with a wash buffer after the feedstream is contacted with the apatite chromatography medium but before eluting the rAAV particles from the apatite chromatography medium.

17. The method of claim 16, wherein the apatite chromatography medium is washed one or more times with a wash buffer containing about 7.5% (w/v) PEG and/or a wash buffer containing about 5% (w/v) PEG.

18. The method of claim 17, wherein the apatite chromatography medium is further washed with a wash buffer containing less than about 3% (w/v) PEG and/or a wash buffer containing no PEG.

19. The method of claim 16, wherein the wash buffer comprises a buffer selected from the group consisting of borate, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and Tris-HCl.

20. The method of claim 16, wherein the wash buffer has a basic pH.

21. The method of claim 20, wherein the wash buffer comprises borate at a pH between about 8.0 and about 10.0.

22. The method of claim 21, wherein the wash buffer comprises borate at a pH of about 8.0.

23. The method of claim 21, wherein the wash buffer comprises borate at a pH of about 9.0.

24. The method of claim 21, wherein the wash buffer comprises borate at a pH of about 10.0.

25. The method of claim 20, wherein the wash buffer further comprises between 100 and 500 mM of a phosphate.

26. The method of claim 20, wherein the wash buffer further comprises between 50 and 250 mM NaCl.

27. The method of claim 1, wherein the rAAV particles bound to the apatite chromatography medium are eluted with an elution buffer containing low concentrations of PEG or in the absence of PEG.

28. The method of claim 27, wherein the elution buffer comprises a buffer selected from the group consisting of borate, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and Tris-HCl at neutral pH.

29. The method of claim 27, wherein the elution buffer contains less than about 3% (w/v) PEG6000.

30. The method of claim 29, wherein the elution buffer further comprises less than 100 mM phosphate.

31. The method of claim 30, wherein the elution buffer further comprises 50 mM phosphate.

32. The method of claim 31, wherein the elution buffer further comprises between 50 and 250 mM NaCl.

33. A method for isolating a population of recombinant adeno-associated virus (rAAV) particles from in-process impurities in a feedstream, comprising the steps of:

(a) contacting a feedstream containing the rAAV particles with a hydrophobic interaction chromatography (HIC) medium in a high salt buffer, wherein the rAAV particles and the in-process impurities bind to the HIC medium; and

(b) eluting the rAAV particles bound to the HIC medium with a medium salt buffer.

34. The method of claim 33, wherein the HIC medium is selected from the group consisting of Tosoh Butyl 650M, Tosoh SuperButyl 650C, Tosoh Phenyl 650C, EMD Fractogel Phenyl, and Tosoh Has(butyl) resin.

35. The method of claim 33, wherein the high salt buffer comprises between about 0.5 M and about 2.0 M citrate.

36. The method of claim 35, wherein the high salt buffer further comprises between about 1 and about 100 mM phosphate.

37. The method of claim 33, wherein the medium salt buffer comprises less than 0.5 M citrate.

38. The method of claim 37, wherein the medium salt buffer further comprises between about 1 and about 100 mM phosphate.

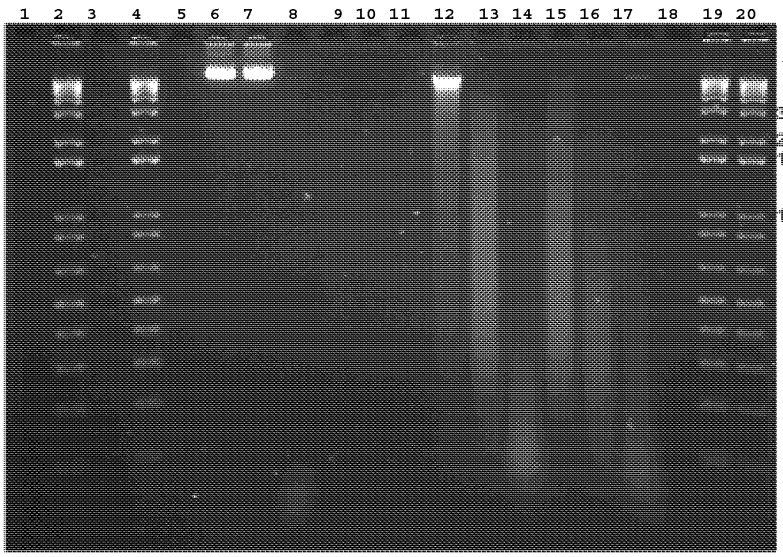
39. The method of claim 37, wherein the medium salt buffer comprises 0.2 M to 0.5 M citrate.

40. The method of claim 39, wherein a population of rAAV particles with empty capsids, partially denatured capsids, less infectious capsid material, and/or partially full capsids are bound to the HIC medium after the elution with the medium salt buffer.

41. The method of claim 1 or claim 33, wherein the rAAV particles comprise an AAV capsid protein from an AAV capsid serotype selected from the group consisting of AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13, AAV-14, AAV-15 and AAV-16.

42. The method of claim 41, wherein the rAAV particles comprise an AAV capsid protein from an AAV capsid serotype selected from the group consisting of AAV-1, AAV-4, AAV-5, and AAV-8.

Figure 1



Lane	Sample
1	Buffer only
2	Ladder in TE
3	-
4	Ladder in post-clarification feedstream (PCLF)
5	-
	Controls
6	λ DNA in PCLF (no Benz.)
7	stop control (λ +PCLF+EDTA+Benz.)
8	positive control (λ DNA in buffer + Benz.)
9	PCLF from production lot
10	Benzonase sample from production lot
11	AEX FT sample from production lot
	Spike digests
12	5 min at 15°C
13	20 min at 15°C
14	62 min at 15°C
15	5 min at 37°C
16	20 min at 37°C
17	62 min at 37°C
18	-
19	Ladder in PCLF
20	Ladder in TE

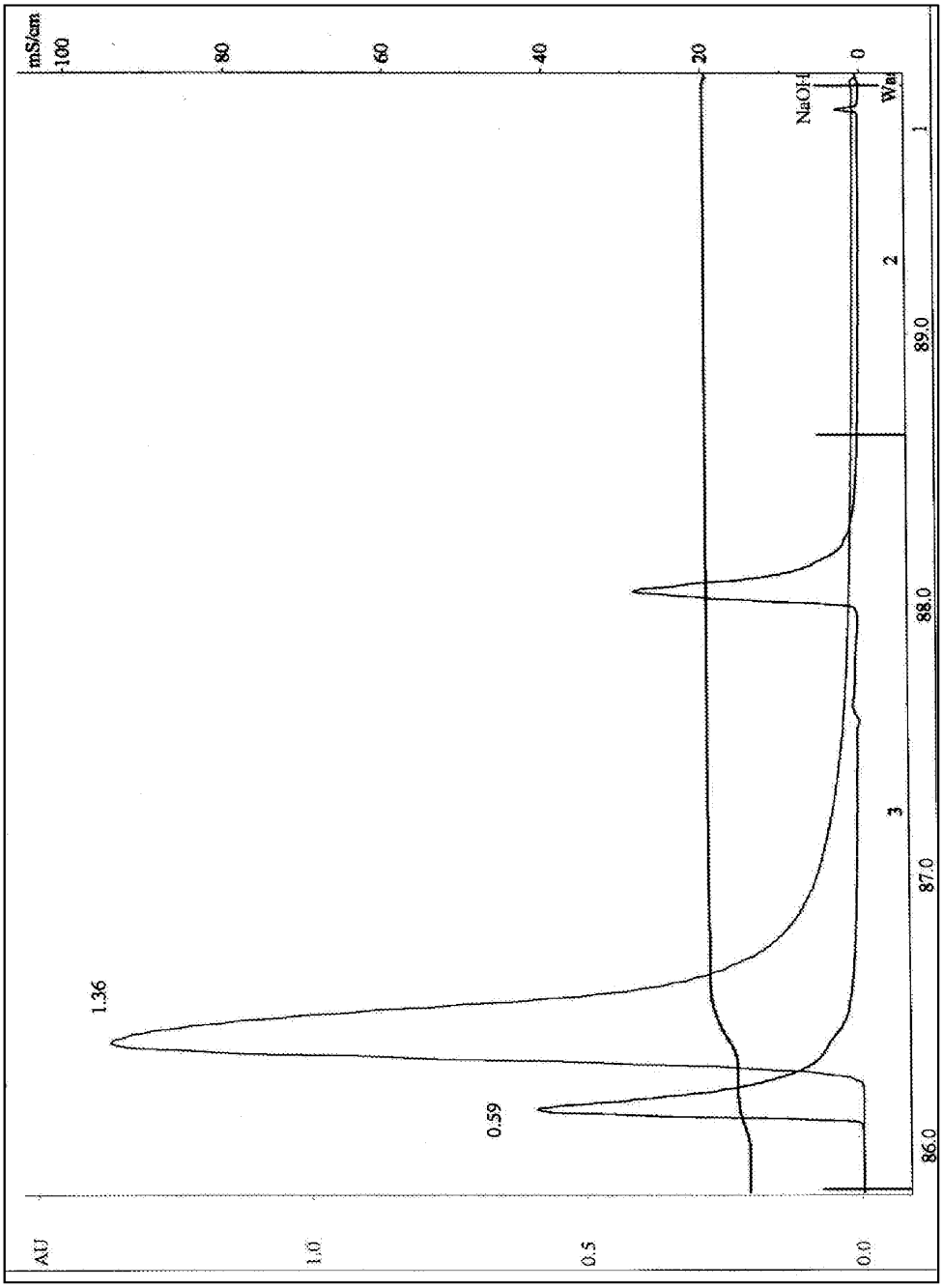
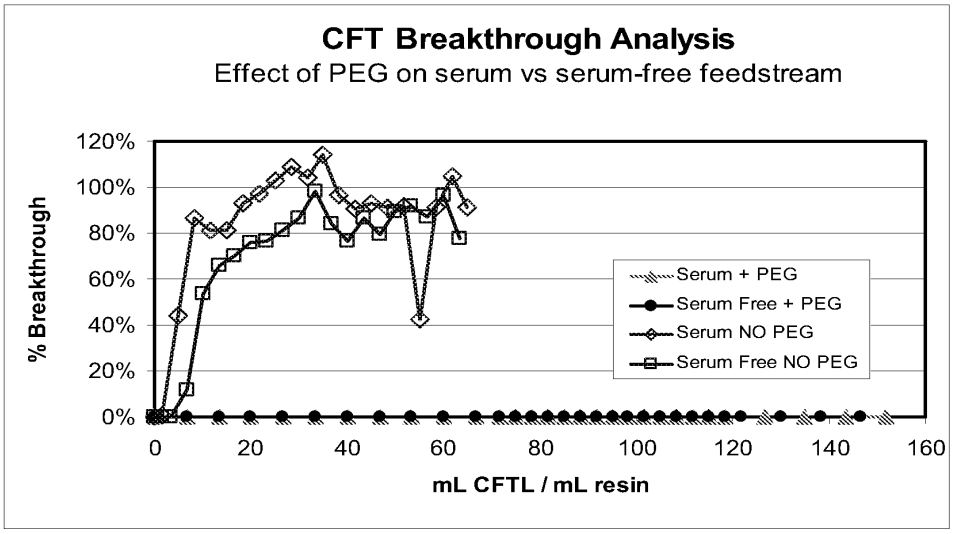

mS/cm
100
80
60
40
20
0
NaOH
Wa
1
2
3
89.0
88.0
87.0
86.0
1.36
0.59
AU
1.0
0.5
0.0

Figure 2

Figure 3



	Load ratio for 1% breakthrough	% Recovered in Elution
Serum NO PEG	~ 2	NA
Serum Free NO PEG	~ 6	NA
Serum + PEG	>150	71%
Serum Free + PEG	>150	69%

Figure 4

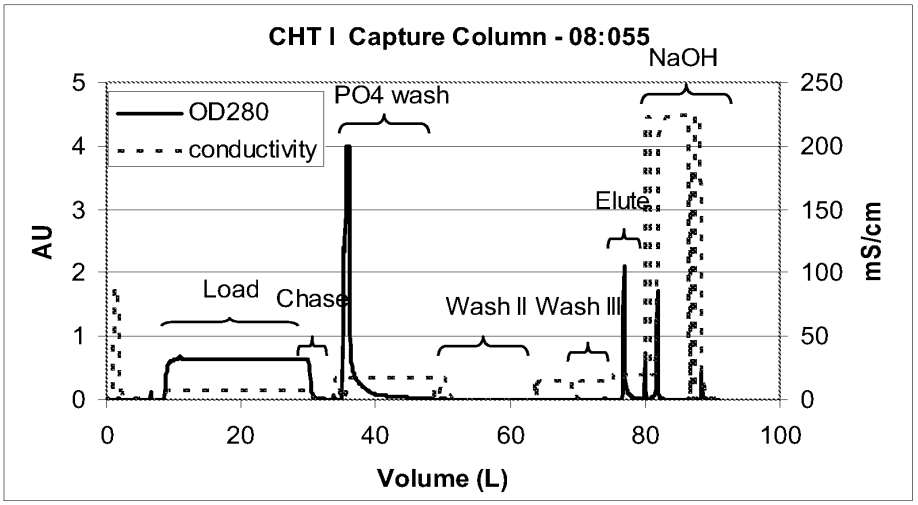
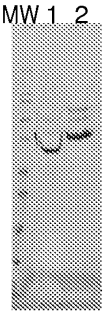

CHT I Capture Column - 08:055
NaOH
PO4 wash
OD280
conductivity
Elute
Load
Chase
Wash II
Wash III
AU
mS/cm
Volume (L)
0
20
40
60
80
100
1
2
3
4
5
50
150
200
250

Figure 5

A

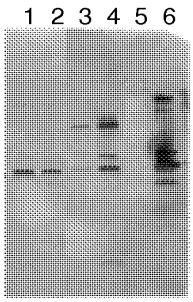
Fraction	CFT	CHT
FT	0%	0%
PO4	1%	2%
WII/III	0%	0%
Elution	**71%**	**65%**
Mass Balance	72%	68%

B



MW - Mark 12
1. CFT elution
2. CHT elution

C



1. CFT elution (1E9 DRP)
2. CHT elution (1E9 DRP)
3. Ad5 (3E7 DRG)
4. Ad5 (3E8 DRG)
5. See Blue MW
6. Capture load (1E8 DRP)

Figure 6

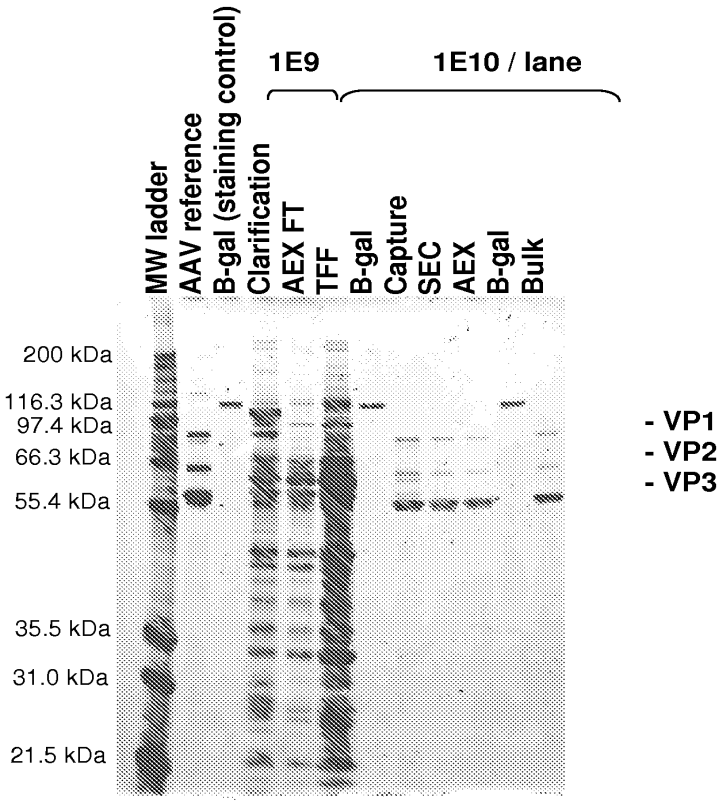
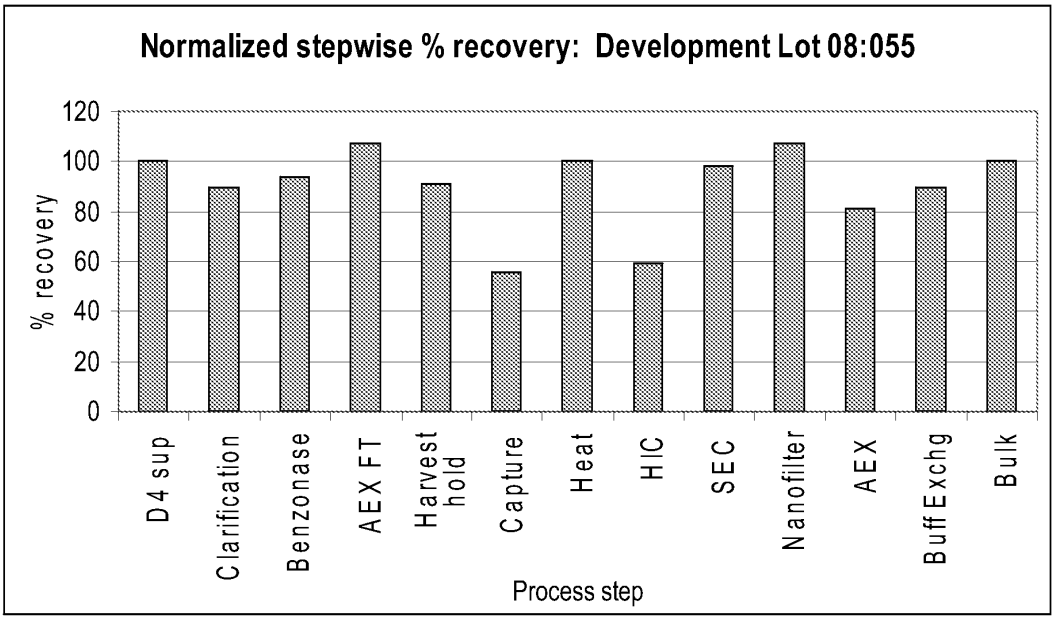

1E9
1E10 / lane
MW ladder
AAV reference
B-gal (staining control)
Clarification
AEX FT
TFF
B-gal
Capture
SEC
AEX
B-gal
Bulk
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
35.5 kDa
31.0 kDa
21.5 kDa
- VP1
- VP2
- VP3

Figure 7


Normalized stepwise % recovery: Development Lot 08:055
% recovery
120
100
80
60
40
20
0
D4 sup
Clarification
Benzonase
AEX FT
Harvest hold
Capture
Heat
HIC
SEC
Nanofilter
AEX
Buff Exchg
Bulk
Process step

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38897

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 7/02 (2010.01)
USPC - 435/239
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/239

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/239, 235.1 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases searched: PubWEST (PGPB, UPST, EPAB, JPAB); Google Scholar
Search terms: Virus purification,virus particles, chromatographic purification, polyethylene glycol (PEG) in mobile phase, hydroxyapatite (HAP, CHT, CFT), hydrophobic interaction chromatography (HIC), recombinant adeno-associated virus (rAAV), adenovirus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	CHAHAL et al. Primary recovery and chromatographic purification of adeno-associated virus type 2 produced by baculovirus/insect cell system. J Virol Meth January 2007 Vol 139 No 1 Pages 61-70. Especially pg 62 right col para 2 and 3, pg 66 right col para 2, pg 67 table 3, pg 69 left col para 1.	33,34,41 ---------- 35-40,42
Y	US 7,015,026 B2 (O'RIORDAN et al.) 21 March 2006 (21.03.2006). Especially col 4 ln 33-38,col 11 ln 25-26, col 12 ln 33-37, col 18 ln 33-41, sheet 9 fig 9A,B,	1-32
Y	GAGNON et al. Method for obtaining unique selectivities in ion-exchange chromatography by addition of organic polymers to the mobile phase. J Chromatog A 30 August 1996 Vol 743 No 1 Pages 51-55. Especially pg 52 left col para 4 and right col para 4,	1-32
Y	BUROVA et al.Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications. Gene Ther October 2005 Vol 12 Suppl 1 Pages S5-S17. Especially pg S10 table 1.	4
Y	URABE et al. Removal of Empty Capsids from Type 1 Adeno-Associated Virus Vector Stocks by Anion-Exchange Chromatography Potentiates Transgene Expression. Mol Ther April 2006 Vol 13 No 4 Pages 823-828. Especially pg 823 right col para 2, pg 824 left col para 3, pg 824 right left col para 3, pg 824 right col para 2,	8,9,42
Y	WO 2008/113011 A2 (VEDVICK et al.) 18 September 2008 (18.09.2008). Especially pg 5 ln 3-5 and ln 23-30, pg 6 ln 1-2, pg 33 ln 2, pg 33 ln 19-20.	35-40

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2010 (24.07.2010)	17 AUG 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
159792010340		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2016/013947	19 January 2016 (19-01-2016)	20 January 2015 (20-01-2015)
Applicant		
GENZYME CORPORATION		

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

This international search report consists of a total of 3 sheets.

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

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

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

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

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

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

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. 1b
      [X] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [ ] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/013947

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/02 G01N9/30
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Ewoud Van Tricht ET AL: "MSc Chemistry Analytical Sciences Virus Particle Characterization Techniques to Quantify Virus Particle Aggregation and Integrity", , 1 July 2013 (2013-07-01), pages 1-51, XP055258342, Retrieved from the Internet: URL:https://esc.fnwi.uva.nl/thesis/centraal/files/f318319369.pdf [retrieved on 2016-03-15] Whole doc., in particular p.23-24 (esp. Fig.7); p.44 (bottom para.)-45; Table 2 (p.46); Table 3 (p.48). ----- -/--	1-95

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2016	12/04/2016

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

2

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

Novartis Exhibit 1002, Page 447 of 591

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERKOWITZ ET AL: "Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 362, no. 1, 1 March 2007 (2007-03-01) , pages 16-37, XP022056699, ISSN: 0003-2697, DOI: 10.1016/J.AB.2006.11.031 Whole doc., in partic. abstract; p.17; p.18(col.2, l. 19-21); Fig.8C; p.31, col.1	1-95
X	XIAOYU YANG ET AL: "Determination of Particle Heterogeneity and Stability of Recombinant Adenovirus by Analytical Ultracentrifugation in CsCl Gradients", JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 97, no. 2, 1 February 2008 (2008-02-01), pages 746-763, XP055258325, WASHINGTON, US ISSN: 0022-3549, DOI: 10.1002/jps.21008 Whole doc., in partic. abstract; p.749, col.2; p.751, col.2; p.753	1-95
A	J. FRASER WRIGHT: "Product-Related Impurities in Clinical-Grade Recombinant AAV Vectors: Characterization and Risk Assessment", BIOMEDICINES, vol. 2, no. 1, 3 March 2014 (2014-03-03), pages 80-97, XP009183697, Whole doc., in partic. abstract; table 2 (p.85)	1-95
X,P	BRENDA BURNHAM ET AL: "Analytical Ultracentrifugation as an Approach to Characterize Recombinant Adeno-Associated Viral Vectors", HUMAN GENE THERAPY METHODS, vol. 26, no. 6, 1 December 2015 (2015-12-01), pages 228-242, XP055258343, ISSN: 1946-6536, DOI: 10.1089/hgtb.2015.048 Whole doc. - applicants scientific disclosure of invention.	1-95

2

Electronic Acknowledgement Receipt	
EFS ID:	32269646
Application Number:	15544498
International Application Number:	
Confirmation Number:	2986
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Mary Chacon
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	159792010300
Receipt Date:	06-APR-2018
Filing Date:	18-JUL-2017
Time Stamp:	18:07:43
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	159792010300_SB08_APR2018.pdf	128085 3758b4650c5fce10b0373ebc8f54cd39d865da63	no	4

Warnings:

Information:					
This is not an USPTO supplied IDS fillable form					
2	Foreign Reference	WO10148143A1.pdf	3332741 27813d6e7944b44ac982e299c93cc677da2ef7e3	no	63
Warnings:					
Information:					
3	Non Patent Literature	BERKOWITZ_2007_16-37.pdf	900120 5c22d4a1e2cb7a4fc072753b7b38f5611ff33dcb	no	22
Warnings:					
Information:					
4	Non Patent Literature	BO_2015_119-125.pdf	2161466 2c8b4cfbcd51325279c19263b79402174d53099d	no	7
Warnings:					
Information:					
5	Non Patent Literature	BURNHAM_2015_228-242.pdf	1542488 35ee87504292acf029cecc63e2010e93e85fe496	no	15
Warnings:					
Information:					
6	Non Patent Literature	COLE_1999_163-176.pdf	1011938 16c69ef5bca04030661b5a9e83027b9bd6f74b7c	no	14
Warnings:					
Information:					
7	Non Patent Literature	COLE_2008_143-179.pdf	774790 8247ff179d9e9faab36ae19c90f7ce44a546e454	no	33
Warnings:					
Information:					
8	Non Patent Literature	FURST_1997_307-310.pdf	342609 3f9bf59bd2e9c027cc72b288e319af763e522df1	no	4
Warnings:					
Information:					

9	Non Patent Literature	GOINS_2014_63-79.pdf	3219155 f167b57d653c46095e61c06150f2e6736de19d85	no	17
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Information:					
10	Non Patent Literature	ISR_12042016_PCT-013947_3pgs.pdf	3005732 732cce6a011f3424436e8cf92369c1654c782fdd	no	3
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Information:					
11	Non Patent Literature	KAPRANOV_2012_46-55.pdf	519405 b6336d9c4930abcf405b5c69b2f812869dfa6792	no	10
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Information:					
12	Non Patent Literature	MCCARTY_2001_1248-1254.pdf	3294188 d538594b6bfc433fe1b1614fd1eb70559dfa789b	no	7
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Information:					
13	Non Patent Literature	QU_2007_183-192.pdf	665603 fcddd44f177fa2919231e132f7f741b11854b017	no	10
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Information:					
14	Non Patent Literature	SCHUCK_2000_1606-1619.pdf	349471 8e2d16623b14b74fcb0f09d83e5dca7c1bb255ec	no	14
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Information:					
15	Non Patent Literature	SEGURA_2013_987-1011.pdf	718331 26ce096ded1f582e6cd3caf7f641dc81f6db0d1a	no	26
Warnings:					
Information:					

16	Non Patent Literature	SOMMER_2003_122-128.pdf	220833 2e706017025eb3da1438460858cb13401abdd462	no	7
Warnings:					
Information:					
17	Non Patent Literature	VAN-TRICHT_2013_51pgs.pdf	1247923 7ddd742dba96642b8b9f26e7b0efe679dbfb0c12	no	51
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
18	Non Patent Literature	WRIGHT_2014_80-97.pdf	284583 fe7b345f4422b06b3b75655177e45a58f360b166	no	18
Warnings:					
Information:					
19	Non Patent Literature	XIAO_1998_2224-2232.pdf	1372601 75e661265b73a9d438a5f07086a88b5c9ef2ce46	no	9
Warnings:					
Information:					
20	Non Patent Literature	YANG_2008_746-763.pdf	1894866 7e02d441144e16bfe7b83cb8e00a2572bbd1e1a5	no	18
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Information:					
		Total Files Size (in bytes):	26986928		

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New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 15979-20103.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patents Application of:
Catherine R. O'RIORDAN et al.

Application No.: 15/544,498

Filed: January 19, 2016 (Int'l)

For: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Confirmation No.: 2986

Art Unit: 1648

Examiner: Not Yet Assigned

PRELIMINARY AMENDMENT UNDER 37 C.F.R. 1.115

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

Dear Commissioner:

INTRODUCTORY COMMENTS

Prior to examination on the merits, Applicant respectfully requests entry of this Preliminary Amendment for the above-captioned patent application. This Amendment is filed as part of the response to Notification of Insufficiency, dated March 23, 2018, for which a response was due May 23, 2018. Applicant hereby petitions for an extension of time to extend the response from May 23, 2018 to June 23, 2018. Since June 23, 2018 landed on a Saturday, the action may be taken on the next succeeding business day. Accordingly, this response is timely filed.

Amendments to the Claims are reflected in the listing of claims which begins on page **2** of this paper.

Remarks/Arguments begin on page **11** of this paper.

pa-1841641

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

Claim 1 (Original): A method of characterizing a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral recombinant viral particle.

Claim 2 (Original): A method to assess vector genome integrity of recombinant viral particles in a preparation of recombinant viral particles comprising

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and

c) identifying species of recombinant viral particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant viral recombinant viral particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant viral particles comprising encapsidated viral genomes of known nucleotide sizes.

Claim 3 (Currently Amended): The method of claim 2 further comprising integrating the area under each peak in the C(s) [[C(S)]] distribution to determine the relative concentration of each species of recombinant viral recombinant viral particles.

Claim 4 (Original): A method to determine the presence of empty capsids or capsid particles comprising variant sized recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals, and

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

Claim 5 (Currently Amended): A method of measuring the relative amount empty capsids in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) [[C(S)]] distribution to determine the relative concentration of each species of recombinant viral particles, and

d) comparing the amount of recombinant viral particles having an S value corresponding to empty capsid particles to the amount of recombinant viral particles having an S value corresponding to recombinant viral particles comprising intact viral genomes or the total amount of recombinant viral particles in the preparation.

Claim 6 (Currently Amended): A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) [[C(S)]] distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value that corresponds to recombinant viral particles comprising intact viral genomes or to the total amount of recombinant viral particles in the preparation.

Claim 7 (Currently Amended): A method of measuring the relative amount of capsid particles comprising variant recombinant viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) [[C(S)]] distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values that do not correspond to recombinant viral particles comprising intact viral genomes or empty capsid particles to the total amount of recombinant viral particles in the preparation.

Claim 8 (Currently Amended): A method of measuring the relative amount of recombinant viral particles comprising intact viral genomes in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) [[C(S)]] distribution to determine the relative concentration of each species of recombinant viral particles,

d) comparing the amount of recombinant viral particles having an S values corresponding to recombinant viral particles comprising intact viral genomes to the amount of recombinant viral particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant viral genomes, and/or to the total amount of recombinant viral particles in the preparation.

Claim 9 (Currently Amended): A method of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant viral genomes during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant viral genomes according to the method of claim 5, ~~any one of claims 5-8,~~ wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant viral particles.

Claim 10 (Currently Amended): The method of claim 4, ~~any one of claims 4-9,~~ wherein the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles; or the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes.

Claim 11 (Canceled)

Claim 12 (Currently Amended): The method of claim 10, [[11,]] wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant viral genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes, and wherein the capsid particles comprising variant sized genomes comprises truncated genomes, aggregates, recombinants and/or DNA impurities.

Claim 13 (Original): A method of determining the heterogeneity of recombinant viral particles in a preparation of recombinant viral particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact viral genome indicates heterogeneity of recombinant particles in the preparation.

Claim 14 (Original): The method of claim 13, wherein the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant viral particles comprising variant genomes.

Claim 15 (Original): The method of claim 14, wherein the variant genomes are truncated viral genomes, aggregates, recombinants and/or DNA impurities.

Claim 16 (Currently Amended): The method of claim 13, ~~any one of claims 13-15~~ further comprising integrating the area under each peak in the C(s) [[C(S)]] distribution to determine the relative concentration of each species of recombinant viral particles.

Claim 17 (Original): A method of monitoring the homogeneity of recombinant viral particles during the purification of a preparation of recombinant viral particles, the method comprising removing a sample of the recombinant viral particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant viral particles according to the method of claim 16, wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes indicates an increase in the homogeneity of full viral particles in the preparation of recombinant viral particles.

Claim 18 (Currently Amended): The method of claim 1, ~~any one of claims 1-17,~~ wherein the sedimentation of recombinant viral particles is monitored by absorbance or interference.

Claims 19-22 (Canceled)

Claim 23 (Currently Amended): The method of claim 1, ~~any one of claims 1-22,~~ wherein the preparation is an aqueous solution.

Claims 24-29 (Canceled)

Claim 30 (Currently Amended): The method of claim 23, ~~any one of claims 24-29,~~ wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles.

Claim 31 (Currently Amended): The method of claim 1, ~~any one of claims 1-30,~~ wherein the C(s) [[C(S)]] values are determined by an algorithm that comprises Lamm equation solutions.

Claim 32 (Original): The method of claim 31, wherein the algorithm is the SEDFIT algorithm.

Claim 33 (Currently Amended): The method of claim 1, ~~any one of claims 1-32,~~ wherein: sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge.

Claim 34 (Currently amended): The method of claim 1~~any one of claims 1-32~~, wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell.

Claim 35 (Currently amended): The method of claim 1~~any one of claims 1-33~~, wherein sedimentation is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell.

Claim 36 (Original): The method of claim 34, wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears an optical window.

Claim 37 (Currently amended): The method of claim 33~~any one of claims 33-36~~, wherein at least 30 scans are used to monitor sedimentation of recombinant viral particles.

Claims 38-41 (canceled)

Claim 42 (Currently amended): The method of claim 31 ~~any one of claims 31-41~~, wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68.

Claim 43 (Original): The method of claim 42, wherein the regularization is a second derivative regularization.

Claim 44 (Original): The method of claim 42, wherein the regularization is Max entropy regularization.

Claims 45-47 (Canceled)

Claim 48 (Currently amended): The method of claim 31~~any one of claims 31-47~~, wherein the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software.

Claims 49-57 (Canceled)

Claim 58 (Currently amended): The method of claim 1, ~~any one of claims 1-57,~~ wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds.

Claims 59-60 (Canceled)

Claim 61 (Currently amended): The method of claim 1, ~~any one of claims 1-60,~~ wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm and/or at about 4°C to about 20 °C.

Claims 62-66 (Canceled)

Claim 67 (Currently amended): The method of claim 1, ~~any one of claims 1-66,~~ wherein the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle.

Claim 68 (Currently amended): A method of evaluating a process for the production of recombinant viral particles comprising the method of claim 1, ~~any one of claims 1 to 67,~~ wherein an increase in the relative amount of recombinant viral particles comprising intact viral genomes compared to the relative amount of empty capsid particles and/or recombinant viral capsid particles with variant recombinant viral genomes compared to a reference preparation of recombinant viral particles indicates an improvement in the production of recombinant viral particles.

Claims 69-79 (Canceled)

Claim 80 (Currently amended): A method for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising

a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant viral particles;

c) isolating the recombinant viral particles produced by the host cell; and

d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the method of claim 1 ~~methods of any one of claims 1 to 79~~.

Claims 81-95 (Canceled)

REMARKS

By virtue of this amendment, claims 3, 5-10, 12, 16, 18, 23, 30-31, 33, 37, 42, 48, 58, 61, 67-68, and 80 have been amended and claims 11, 19-22, 24-29, 38-41, 45-47, 49-57, 59-60, 62-66, 69-79, and 81-95 been canceled. Accordingly, claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 67-68, and 80 are currently under consideration.

Support for the claim amendments may be found throughout the specification. Claims 3, 5-8, 16, and 31 have been amended to correct typographical errors. Claims 9-10, 12, 16, 18, 23, 30-31, 33, 37, 58, 61, 67-68, and 80 have been amended to remove multiple dependencies and/or to update dependencies following claim cancelation. Claim 10 has also been amended to optionally recite the limitations of canceled claim 11. Claim 12 has also been amended to recite the limitations of canceled claim 11. Claim 18 has also been amended to optionally recite the limitations of canceled claim 21. Claim 61 has also been amended to optionally recite the limitations of canceled claim 65. No new matter is believed to have been added.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20103.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: June 25, 2018

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
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Electronic Patent Application Fee Transmittal	
Application Number:	15544498
Filing Date:	18-Jul-2017
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Filer:	Brian Arthur Donahue/Aurelia Caparas
Attorney Docket Number:	15979-20103.00
Filed as Large Entity	

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1615	15	100	1500
INDEPENDENT CLAIMS IN EXCESS OF 3	1614	7	460	3220
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**4920**

Electronic Acknowledgement Receipt	
EFS ID:	32992533
Application Number:	15544498
International Application Number:	
Confirmation Number:	2986
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20103.00
Receipt Date:	25-JUN-2018
Filing Date:	18-JUL-2017
Time Stamp:	19:00:15
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$4920
RAM confirmation Number	062618INTEFSW00006815031952
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

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	Copy_Notification_Insufficiency.pdf	146296 1151a5fc848cfbf812b127fd5ddad80b4ea4c08c	no	2
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Information:					
2		2nd_prelim_for_resp_to_MP.pdf	117604 b1789af53ffde336547176a686bff8aa959294a5	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Claims	2	10
	Applicant Arguments/Remarks Made in an Amendment	11	12

Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	34451 35ad8a4137ca12fc7805340500c3795b2a995491	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	298351		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
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U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
15/544,498	Catherine R. O'RIORDAN	159792010300

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

INTERNATIONAL APPLICATION NO.	
PCT/US2016/013947	
I.A. FILING DATE	PRIORITY DATE
01/19/2016	01/20/2015

CONFIRMATION NO. 2986
371 FORMALITIES LETTER

OC000000098242672

Date Mailed: 03/23/2018

Notification of Insufficiency Under 37 CFR 1.492 and/or 1.495 (DO/EO/US)

The following items have been submitted by the applicant or the International Bureau to the United States Patent and Trademark Office as a Designated / Elected Office (37 CFR 1.495).

- Priority Document
- Copy of the International Application filed on 07/18/2017
- Copy of the International Search Report filed on 07/18/2017
- Preliminary Amendments filed on 07/18/2017
- Inventor's Oath or Declaration filed on 07/18/2017
- U.S. Basic National Fees filed on 07/18/2017
- Authorize Access to Search Results filed on 07/18/2017
- Priority Documents filed on 07/18/2017
- Power of Attorney filed on 07/18/2017
- Specification filed on 07/18/2017
- Claims filed on 07/18/2017
- Abstracts filed on 07/18/2017
- Drawings filed on 07/18/2017
- Authorization to Permit Access filed on 07/18/2017
- Application Data Sheet (37 CFR 1.76) filed on 07/18/2017

The following items **MUST** be furnished within the period set forth below:

- Additional claim fees of $**13240** as a undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**13240** for an Undiscounted Entity:

Total additional claim fee(s) for this application is $**13240**
- $**3220** for **7** independent claims over 3.
- $**9200** for **92** total claims over 20.
- $**820** for multiple dependent claim surcharge.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

FORM PCT/DO/EO/923 (371 Formalities Notice)

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

BRIAN A HARDEN

Telephone: (703) 756-1815

FORM PCT/DO/EO/923 (371 Formalities Notice)

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/06/2018

BHARDEN ADJ #00000002 Mailroom Dt: 06/25/2018
Seq No: 6815 Sales Acctg Dt: 06/26/2018 031952 15544498
02 FC : 1614 3220.00 CR

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/06/2018

BHARDEN SALE #00000002 Mailroom Dt: 06/25/2018 031952 15544498
01 FC : 1614 2,760.00 DA

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/544,498	07/18/2017	Catherine R. O'RIORDAN	15979-20103.00

CONFIRMATION NO. 2986

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

PUBLICATION NOTICE

OC000000100620982

Title:ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Publication No.US-2018-0180525-A1
Publication Date:06/28/2018

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/544,498

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	35 minus 20 =	* 15			OR	x 100 =	1500
INDEPENDENT CLAIMS (37 CFR 1.16(h))	9 minus 3 =	* 6				x 460 =	2760
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	6240

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
15/544,498	Catherine R. O'RIORDAN	15979-20103.00

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

INTERNATIONAL APPLICATION NO.	
PCT/US2016/013947	
I.A. FILING DATE	PRIORITY DATE
01/19/2016	01/20/2015

CONFIRMATION NO. 2986
371 ACCEPTANCE LETTER

OC000000100797529

Date Mailed: 07/10/2018

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

07/18/2017
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 07/18/2017
- Copy of the International Search Report filed on 07/18/2017
- Preliminary Amendments filed on 07/18/2017
- Inventor's Oath or Declaration filed on 07/18/2017
- U.S. Basic National Fees filed on 07/18/2017
- Authorize Access to Search Results filed on 07/18/2017
- Priority Documents filed on 07/18/2017
- Power of Attorney filed on 07/18/2017
- Specification filed on 07/18/2017
- Claims filed on 07/18/2017
- Abstracts filed on 07/18/2017
- Drawings filed on 07/18/2017
- Authorization to Permit Access filed on 07/18/2017
- Application Data Sheet (37 CFR 1.76) filed on 07/18/2017

FORM PCT/DO/EO/903 (371 Acceptance Notice)

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

BRIAN A HARDEN

Telephone: (703) 756-1815

FORM PCT/DO/EO/903 (371 Acceptance Notice)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
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www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/544,498	07/18/2017	1648	6140	15979-20103.00	35	9

CONFIRMATION NO. 2986

UPDATED FILING RECEIPT

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

OC000000100797528

Date Mailed: 07/10/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Catherine R. O'RIORDAN, Bridgewater, NJ;
Brenda BURNHAM, Hopkinton, MA;

Applicant(s)

Genzyme Corporation, Cambridge, MA;

Assignment For Published Patent Application

Genzyme Corporation, Cambridge, MA
Genzyme Corporation, Cambridge, MA

Power of Attorney: The patent practitioners associated with Customer Number 89300

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2016/013947 01/19/2016
which claims benefit of 62/105,714 01/20/2015

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 03/22/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/544,498**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

Title

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

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UNITED STATES DEPARTMENT OF COMMERCE
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/544,498	07/18/2017	Catherine R. O'RIORDAN	15979-20103.00	2986

89300 7590 08/16/2018

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CALIFORNIA 94304
UNITED STATES OF AMERICA

EXAMINER
GILL, RACHEL B

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
08/16/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

PTOL-90A (Rev. 04/07)

Office Action Summary	**Application No.** 15/544,498	**Applicant(s)** O'RIORDAN et al.	
	Examiner RACHEL B GILL	**Art Unit** 1648	**AIA Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 06 April 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-10,12-18,23,30-37,42-44,48,58,61,67-68 and 80 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-10,12-18,23,30-37,42-44,48,58,61,67-68 and 80 is/are rejected.

8) ☑ Claim(s) 1 is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 18 July 2017 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Disposition of Claims

Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 67-68, and 80 are pending.

Examiner's Note

All paragraph numbers (¶) throughout this office action, unless otherwise noted, are from the US PGPub of this application 2018/0180525 A1, Published 06/28/2018.

The Examiner would like to note that interview requests are welcomed by the Office, but a request for an in-person interview at the Alexandria Office location must be made in a timely fashion prior to the interview, as the Examiner is not currently located at the Alexandria Office. Applicant is encouraged to utilize the new web-based Automated Interview Request (AIR) tool for submitting interview requests; more information can be found at https://www.uspto.gov/patent/laws-and-regulations/interview-practice.

Optional Authorization to Initiate Electronic Communications

The Applicant's representative may wish to consider supplying a written authorization in response to this Office action to correspond with the Examiner via electronic mail (e-mail). This authorization is **optional** on the part of the Applicant's representative, but it should be noted that the Examiner may not initiate nor respond to communications via electronic mail unless and until Applicant's representatives authorize such communications in writing within the official record of the patent application. A sample authorization is available at MPEP § 502.03, part II.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on 04/06/2018 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

Claim Objections

Claim 1 is objected to because of the following informalities: "recombinant viral" is repeated in lines 9-10. Appropriate correction is required.

Claim Rejections - 35 USC § 112(b); Second Paragraph

> The following is a quotation of 35 U.S.C. 112(b):
> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 12 and 15 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

The claims provide for variant genomes which may be truncated genomes, aggregates, DNA impurities, or recombinants. However, it is not clear how the recombinant variant genomes differ from the "true" recombinant genomes from which the claims depend.

For at least these reasons, claims 12 and 15 are rejected on the grounds of being indefinite.

Claim Rejections - 35 USC § 102

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –

(a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.

Claims 1, 18, 23, 30-37, 58, and 61 are rejected under 35 U.S.C. 102(a)(1) as being anticipated by Cole (Cole JL. *Biochemistry*. 1996 Dec 3;35(48):15601-10; APPLICANT-CITED PRIOR ART; hereafter "Cole".)

The claims are interpreted as being drawn to the following:

Claim 1 is drawn to a method of characterizing a preparation of recombinant viral particles comprising the steps of:

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle.

Further limitations of the method of claim 1 are wherein the sedimentation of recombinant viral particles is monitored by absorbance or interference (claim 18); wherein the preparation is an aqueous solution (claim 23); wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles (claim 30); wherein the C(s) values are determined by an algorithm that comprises Lamm equation solutions (claim 31); wherein the algorithm is the SEDFIT algorithm (claim 32); wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge (claim 33); wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell (claim 34); wherein sedimentation is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell (claim 35); wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears an optical window (claim 36); wherein at least 30 scans are used to monitor sedimentation of recombinant viral particles (claim 37); wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds (claim

58); and wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm and/or at about 4°C to about 20 °C (claim 61).

The Prior Art

Cole teaches that Analytical ultracentrifugation (AUC) is a versatile and powerful method for the quantitative analysis of macromolecules in solution (abstract) and can be utilized to determine the size and shape of macromolecules in solution, such as viruses (p. 2, ¶2). Note that "recombinant viral particle" is not specifically defined in the specification (¶[0058] appears to be specifically drawn to "recombinant AAV particles"). Furthermore, "recombinant viral vectors" or "recombinant viruses" appear to be defined in the specification as a virus comprising one or more heterologous sequences, such as nucleic acid not of viral origin. However, homologous recombination is a natural process amongst many viruses, such as herpesviruses, and recombine to incorporate genetic material not of herpesvirus origin, i.e. genetic information from other viruses, bacteria, or host cells. Therefore, "recombinant" is an adjective that is not interpreted to provide any meaningful limitation to the term "virus" or "viral particle", and "recombinant viral particles" and "recombinant viruses" are interpreted to be equivalent to "viral particles" and "viruses".

Cole teaches subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (p. 3, ¶1), plotting the differential sedimentation coefficient distribution value (C(s)) (p. 5, ¶3) versus the sedimentation coefficient (s) (p. 3, ¶1) in Svedberg units (S) (p. 3, ¶3-4; p. 4, ¶3; p. 15, ¶2; See "B. Data Analysis" starting on p. 15, Table 4) and integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle (abstract; p. 4, ¶1; p. 5, ¶1; pp. 15-16, ¶ bridging pages; See "B. Data Analysis" starting on p. 15, esp. ¶ bridging pp. 15-16.) Therefore, Cole anticipates the limitations of **instant claim 1**.

Cole teaches the sedimentation from aqueous solutions is monitored by absorbance, interference, or fluorescence (abstract; **instant claims 18, 23**). Cole teaches adjusting the optics to account for the solvent component interference in the sample solution (p. 7, ¶2) and the use of reference solutions in absorbance optics for AUC (p. 7, ¶5; **instant claim 30**). Cole teaches that the C(s) values are determined by an algorithm that comprises Lamm equation solutions (p. 3, ¶2-4; p. 16, ¶3; **instant**

claim 31); wherein the algorithm is the SEDFIT algorithm (p. 12, ¶3; p. 16, ¶ 3; p. 17, ¶2; **instant claim 32**). Cole teaches that sedimentation is monitored according to the sedimentation coefficient of the sample, and should take a boundary at least 2 hours to sediment the full length of a cell to the bottom of a sector (p. 15; ¶2; **instant claim 33**). Cole teaches optimizing solvents according to their compatibility with the sample cell and with optical systems (p. 2, ¶2; **instant claim 34**). Cole describes the instrumentation, and details the rotors, sample holders, and optical systems, including:

"To view the sample, the analytical rotor has holes through it to hold sample containers commonly called cells. Each cell contains a centerpiece, with chambers (called channels) to hold the liquid samples. The centerpiece, in turn, is sealed between windows to permit the passage of light through the channels, thus allowing the cell contents to be viewed."

The instrumentation described would allow monitoring of the sedimentation of particles within the AUC through an optical window (p. 6, ¶2; **instant claim 35-**36). Cole describes the "scans" performed to view the sedimentation (p. 6, ¶3) and states when observing the system using absorbance readings it is best to scan at least 30-40 times (p. 15, ¶2; **instant claim 37**) and depending on the scanning method used, the scans can be performed once every second up to once every 300 seconds (Table 2; **instant claim 58**.) In analyzing the data using SEDFIT, regularization procedures can be performed to produce a smoother distribution function (p, 16, ¶3). Cole describes that depending on what you want to know about your sample and how you are scanning the sample will alter the velocity of the rotor, and that depending on the S (Svedberg units) of the sample, you will use a velocity of anywhere from 10,000-55,000 RPM (Table 3), and with absorbance scanning (especially involving multi-wavelength scanning), one may wish to use a lower speed (Table 3; p. 19, ¶3-4, 6,000-12,000 RPM, p. 7, ¶4) and that larger biomolecules may aggregate or degrade, so one can detect the loss of material by measuring the OD at low speed (3,000 RPM; pp. 12-13, ¶ bridging pages; **instant claim 61**). Cole describes that with some AUCs it is difficult to use the machine with a temperature below 4 °C and that most experiments are performed at about 20 °C (pp. 14-15, ¶ bridging pages; **instant claim 61**).

For at least these reasons, Cole teaches the limitations of claims 1, 18, 23, 30-37, 58, and 61 and anticipates the instant invention.

Claims 1-10, 12-18, 23, 30-36, 61, and 67-68 are rejected under 35 U.S.C. 102(a)(1) as being anticipated by van Tricht (van Tricht E. "Virus Particle Characterization: Techniques to Quantify Virus Particle Aggregation and Integrity." Literature Thesis, MSc Chemistry, Analytical Sciences. Univ. of Amsterdam, 07/2013; APPLICANT-CITED PRIOR ART; hereafter "van Tricht".)

The claims are interpreted as being drawn to the following:

The scope of claims 1, 18, 23, 30-37, 58, and 61 has been set forth *supra*.

Further limitations of the method of claim 1 are wherein the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle (claim 67).

Claims 2-10 and 12-17 are variations on instant claim 1 that assess, via AUC, vector genome integrity of viral particles (claims 2-3), empty capsid presence (claims, 4, 10, 12), empty capsid amounts (claims 5-6), relative amount of variant viral particles (claims 6-7), relative amount of intact viral particles with full genomes (claim 8), removal/lack of empty capsids (claim 9), and/or determination/monitoring of heterogeneity of viral particles (claims 13-17), and/or monitoring production of viral particles (claim 68).

The Prior Art

Van Tricht discloses the assessment of the suitability of several techniques for the characterization of virus particle (in particular adenovirus, see e.g. p. 25, ¶2) aggregation or integrity, including AUC under boundary conditions. Van Tricht teaches subjecting the aqueous preparation (p. 31, ¶2; **instant claim 23**) to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals using absorbance (pp. 19-20, ¶ bridging pages, Fig. 4; **instant claim 18**) and compared to a reference (Fig. 4; **instant claim 30**), plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient (s) in Svedberg units (S) and integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle (Fig. 7.) Therefore, Van Tricht anticipates the limitations of **instant claim 1**. Data analysis using SEDFIT/Lamb is shown just as in present application (see e.g. Fig.7, pp. 23-4 shows monomer v. dimer distribution; **instant claims 31-32**). Van Tricht teaches sedimentation is monitored

within the cell until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge (pp. 19-20, Fig. 4; **instant claims 33-36**).

Van Tricht teaches AUC of adenovirus particles (p. 25, starting at ¶2) wherein said particles were run on a CsCl gradient to have optimal separation between complete (mature) and incomplete (empty capsid) particles based on density differences. Said experiment was performed using absorption monitoring of the CsCl gradient run for 18 hours at 4 deg C at 40,000 RPM (p. 25, **instant claims 61, 67**). Van Tricht teaches separation showed 2 different types of empty capsids and 4 other virus forms (p. 25), and the methods not only assess empty and mature capsids, but also aggregates of virus and proteins. Van Tricht teaches that empty capsids and aggregates should be monitored and removed from manufacturing processes since they can have toxic side effects when used in vaccine preparations (pp. 11-12, pp.13-14, ¶ bridging pages; p. 6, ¶2.) As set forth *supra*, claims 2-10 and 12-17 are variations on instant claim 1 that assess, via AUC, vector genome integrity of viral particles (claims 2-3), empty capsid presence (claims, 4, 10, 12), empty capsid amounts (claims 5-6), relative amount of variant viral particles (claims 6-7), relative amount of intact viral particles with full genomes (claim 8), removal/lack of empty capsids (claim 9), determination/monitoring of heterogeneity of viral particles (claims 13-17) and/or monitoring production of viral particles (claim 68). The methods of Van Tricht therefore teach the methods not only of **instant claim 1**, but also **instant claims 2-10, 12-17, and 68**.

Van Tricht therefore teaches the limitations of instant claims 1-10, 12-18, 23, 30-36, 61, and 67-68, and anticipates the instant invention.

Claim Rejections - 35 USC § 103

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Claims 37, 42-44, 48, and 58 are rejected under 35 U.S.C. 103 as being unpatentable over van Tricht as applied to claims 1-10, 12-18, 23, 30-36, 61, and 67-68 above, and further in view of Cole (*supra*) and Shuck (Schuck P. *Biophys J.* 2000 Mar;78(3):1606-19; APPLICANT-CITED PRIOR ART; hereafter "Shuck".)

The claims are interpreted as being drawn to the following:

The scope of claims 1-10, 12-18, 23, 30-37, 58, 61, and 67-68 have been set forth *supra*.

Further limitations on the instant invention are wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68 (claim 42); wherein the regularization is a second derivative regularization (claim 43); wherein the regularization is Max entropy regularization (claim 44); wherein the following C(s) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software (claim 48); wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds (claim 58);

The Prior Art

The teachings of van Tricht have been set forth *supra*. Van Tricht teaches AUC, but teaches more general methods and parameters for AUC. The teachings of Cole have been set forth *supra*, and render obvious the differences in teachings for van Tricht as far as AUC parameters such as temperature and rotor RPM. Optimization of conditions for the methods of van Tricht would be obvious to one of skill in the art, given the technical teachings of Cole and the further teachings of Shuck. While neither van Tricht nor Cole teach specific regularization methods of calculation, Shuck teaches maximum entropy regularization to represent a continuous size-distribution in analysis of macromolecules by sedimentation velocity ultracentrifugation (entire document; see abstract.) Shuck teaches optimization of sedimentation profiles, such as adjusting the maximum entropy regularization is adjusted to a probability of p = 0.68 with a F ratio (frictional ratio or F statistic) of 1.0 (Fig. 1). Shuck provides teachings and motivation to optimize the parameters of a sediment velocity-based AUC method to separate biomolecules, and the combined teachings of van Tricht, which teach AUC for adenovirus virions, Cole, which teaches the basic principles of AUC, and Shuck, which teach the more technical parameters of data analysis of AUC, would render obvious the limitations of **instant claims 42-44, 48, and 58**, especially through routine optimization one of skill would have to take in order to maximize the separation profiles of empty and mature viral particles.

It would have been obvious to one of ordinary skill in the art to modify the methods taught by van Tricht and Cole in order to optimize the AUC methods, thereby specifically identifying the data refining equations and methods to be used. One would have been motivated to do so, given the suggestion by Shuck that optimization of sedimentation profiles for separation of biomolecules using AUC is essential and routine. There would have been a reasonable expectation of success, given the knowledge as to how to refine the data and protocol for biomolecules separated by AUC, as taught by Shuck. Thus the invention as a whole was clearly *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

Claim 80 is rejected under 35 U.S.C. 103 as being unpatentable over van Tricht as applied to claims 1-10, 12-18, 23, 30-36, 61, and 67-68 above, and further in view of Colosi (US 2009/0017542 A1, Pub. 01/15/2009, hereafter "Colosi".)

The claims are interpreted as being drawn to the following:

The scope of claims 1-10, 12-18, 23, 30-37, 58, 61, and 67-68 have been set forth *supra*.

Claim 80 is drawn to a method for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising:

a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise:

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant viral particles;

c) isolating the recombinant viral particles produced by the host cell; and

d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the method of claim 1.

The Prior Art

Van Tricht teaches the purification of adenoviruses using the method of claim 1, and teaches the feasibility of applying such purification methods to other systems, but is silent as to the purification of adeno-associated virus (AAV), a virus that, like adenovirus, is commonly studied for gene therapy and vaccine purposes.

Colosi teaches a method of producing recombinant AAV (rAAV) virions comprising:

(a) introducing an AAV vector into a suitable host cell;

(b) introducing a AAV helper function vector into the host cell; wherein said vector is comprising:

an AAV rep coding region;

an AAV cap coding region; and

a nucleotide sequence comprising a modified AAV p5 promoter region, such that the modified AAV p5 promoter region no longer functions in transcription initiation;

(c) expressing accessory functions in the host cell; and

(d) culturing the host cell to produce rAAV virions (reference claims 1-2, 5). Colosi teaches that with unlike previously described methods for producing rAAV, the AAV helper function vectors generate high titer stocks of rAAV but do not produce any detectable wild-type AAV (¶[0029]).

Taking the teachings of van Tricht, which teach one of ordinary skill how to use AUC purification methods and teaches how such methods may be applied to other viruses, one of ordinary skill would be motivated to try said method on purifying AAV. Colosi teaches growing recombinant AAV (rAAV), but is silent as to the exact method of collecting the desired rAAV virions. One of skill in the art would turn to the methods available for virion purification, especially one that would allow one of skill to distinguish and separate empty capsids from other types of virions. The method of van Tricht would be an obvious choice to separate the virions grown by Colosi, as AAV is desired for use in gene therapy and vaccine compositions. Therefore, the limitations of **instant claim 80** would be obvious to one of ordinary skill in the art, given the teachings of Colosi and van Tricht.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RACHEL B GILL whose telephone number is (571)272-3129. The examiner can normally be reached on M to F 8:00 AM to 5:00 PM Eastern.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, JANET ANDRES can be reached on 571-272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RACHEL B GILL/
Primary Examiner, Art Unit 1648

Notice of References Cited	Application/Control No. 15/544,498	Applicant(s)/Patent Under Reexamination O'RIORDAN et al.	
	Examiner RACHEL B GILL	Art Unit 1648	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20090017542-A1	01-2009	Colosi; Peter	C07K14/005	435/455
	B					
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FOREIGN PATENT DOCUMENTS

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	Art Unit
	RACHEL B GILL	1648

CPC - Searched*		
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CPC Combination Sets - Searched*		
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Class	Subclass	Date	Examiner

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Search Notes		
Search Notes	Date	Examiner
Google inventor, assignee, and keyword search	08/10/2018	RG
PALM inventor search (see attached)	08/10/2018	"
EAST inventor, assignee, and keyword search (see attached)	08/10/2018	"
PubMed inventor, assignee, and keyword search (see attached)	08/10/2018	"

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/RACHEL B GILL/ Primary Examiner, Art Unit 1648	

Bibliographic Data

Application No: 15/544,498

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /RACHEL B GILL/ (Examiner's Signature) | (Initials)

Title: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/18/2017	424	1648	15979-20103.00
RULE			

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Brenda BURNHAM Hopkinton, MA, UNITED STATES

CONTINUING DATA

This application is a 371 of PCT/US2016/013947 01/19/2016

PCT/US2016/013947 has PRO of 62105714 01/20/2015

FOREIGN APPLICATIONS

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#22	Add	**Similar articles** for **PubMed** (Select **26958574**)	96	09:07:56
#18	Add	Search **(O'Riordan C[Au]) AND burnham b[au]**	4	09:07:43
#17	Add	Search **(genzyme) AND burnham b[au]**	2	09:07:34
#16	Add	Search **(genzyme) AND O'Riordan C[Au]**	19	09:07:30
#15	Add	Search **((genzyme) AND O'Riordan C[Au]) AND burnham b[au]**	2	09:07:26
#14	Add	Search **genzyme**	1697	09:07:20
#13	Add	Search **O'Riordan C[Au]**	57	09:07:11
#12	Add	Search **O'Riordan CR[Author]**	31	09:07:05
#11	Add	Search **O'Riordan CR[AU]**	25	09:07:05
#9	Add	Search **burnham b[au]**	83	09:06:54
#8	Add	**Similar articles** for **PubMed** (Select **8952514**)	95	09:05:46
#7	Add	Search **Cole JL. Characterization of human cytomegalovirus protease dimerization by analytical centrifugation. Biochemistry 1996**	1	09:05:34

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Inventor Name Search Result

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Last Name = BURNHAM
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Application#	Patent#	PG Pub#	Status	Date Filed	Title	Examiner Name	Inventor Name
15544498	Not Issued	20180180525	30	07/18/2017	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	GILL,RACHEL	BURNHAM, BRENDA
62105714	Not Issued		159	01/20/2015	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	,	BURNHAM, BRENDA

Inventor Search Completed: No Records to Display.

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Inventor Name Search Result

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Last Name = O'RIORDAN
First Name = CATHERINE

Application#	Patent#	PG Pub#	Status	Date Filed	Title	Examiner Name	Inventor Name
09029483	Not Issued		157	02/08/1998	VIRAL VECTOR CONSTRUCTS	KAUSHAL,SUMESH	O'RIORDAN, CATHERINE
11934148	Not Issued	20090069261	41	11/02/2007	GENE THERAPY FOR SPINAL CORD DISORDERS	WEHBE,ANNE MARIE	O'RIORDAN, CATHERINE
12328267	9034836	20100267812	150	12/04/2008	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
12417910	9890394	20090286857	150	04/03/2009	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
14692469	Not Issued	20160067312	41	04/21/2015	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
15127757	Not Issued	20170173183	41	09/20/2016	GENE THERAPY FOR RETINITIS PIGMENTOSA	LI,QIAN	O'RIORDAN, CATHERINE
15549825	Not Issued	20180023082	41	08/09/2017	VARIANT RNAi	CHONG,KIMBERLY	O'RIORDAN, CATHERINE
15858303	Not Issued	0	30	12/29/2017	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
60135092	Not Issued		159	02/08/1998	VIRAL VECTOR CONSTRUCTS	NOT,DEFINED	O'RIORDAN, CATHERINE
60827277	Not Issued		159	10/03/2006	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	,	O'RIORDAN, CATHERINE
61045077	Not Issued		159	04/15/2008	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RdCVF)	,	O'RIORDAN, CATHERINE
61969027	Not Issued		159	03/21/2014	GENE THERAPY FOR RETINITIS PIGMENTOSA	,	O'RIORDAN, CATHERINE
62105718	Not Issued		159	01/20/2015	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	,	O'RIORDAN, CATHERINE
62114576	Not Issued		159	02/10/2015	NOVEL NUCLEIC ACIDS FOR IMPROVED GENE DELIVERY	,	O'RIORDAN, CATHERINE
62114578	Not Issued		159	02/10/2015	VARIANT RNAi	,	O'RIORDAN, CATHERINE
62375314	Not Issued		159	08/15/2016	METHODS FOR DETECTING AAV	,	O'RIORDAN, CATHERINE
62561938	Not Issued		20	09/22/2017	COMPOSITIONS AND METHODS OF TREATING HUNTINGTON'S DISEASE	,	O'RIORDAN, CATHERINE
09604349	Not Issued		161	06/27/2000	CHROMATOGRAPHIC PURIFICATION OF ADENOVIRUS	SALIMI,ALI	O'RIORDAN, CATHERINE E.
10470604	7015026	20040152183	250	03/02/2004	PURIFICATION OF ADENOVIRUS AND AAV	SALIMI,ALI	O'RIORDAN, CATHERINE E.
11179865	7579181	20050244942	250	07/12/2005	PURIFICATION OF ADENOVIRUS AND AAV	MOSHER,MARY	O'RIORDAN, CATHERINE E.
12504073	Not Issued	20100279385	161	07/16/2009	PURIFICATION OF ADENOVIRUS AND AAV	MOSHER,MARY	O'RIORDAN, CATHERINE E.
09011828	6143548		150	06/29/1998	CHROMATOGRAPHIC PURIFICATION OF ADENOVIRUS AND AAV	SALIMI,ALI	O'RIORDAN, CATHERINE E.
60071674	Not Issued		159	01/16/1998	ADENOVIRAL VECTORS WITH MODIFIED CAPSID PROTEINS	,	O'RIORDAN, CATHERINE E.
60097653	Not Issued		159	08/24/1998	CATIONIC COMPLEXES OF POLYMER-MODIFIED ADENOVIRUS	,	O'RIORDAN, CATHERINE E.
60183296	Not Issued		159	02/17/2000	Adenoviral system	,	O'RIORDAN, CATHERINE R
09582502	Not Issued		161	06/26/2000	ADENOVIRAL VECTORS WITH MODIFIED CAPSID PROTEINS	GUZO,DAVID	O'RIORDAN, CATHERINE R.
09603022	Not Issued		161	07/24/2000	NUCLEIC ACID DELIVERY VEHICLES	GUZO,DAVID	O'RIORDAN, CATHERINE R.

10349636	Not Issued	20030180261	161	01/22/2003	Polymer-modified viruses	NAFF,DAVID	O'RIORDAN, CATHERINE R.
12936659	9367382	20110034546	150	10/06/2010	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)	BURKHART,MICHAEL	O'RIORDAN, CATHERINE R.
14888385	Not Issued	20160074474	61	02/08/2016	COMPOSITIONS AND METHODS FOR TREATING SPINAL MUSCULAR ATROPHY	SCHULTZ,JAMES	O'RIORDAN, CATHERINE R.
15397455	10035829	20170369541	150	01/03/2017	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)	BURKHART,MICHAEL	O'RIORDAN, CATHERINE R.
15544498	Not Issued	20180180525	30	07/18/2017	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	GILL,RACHEL	O'RIORDAN, CATHERINE R.
16022410	Not Issued		19	06/28/2018	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)	,	O'RIORDAN, CATHERINE R.
61818448	Not Issued		159	05/01/2013	COMPOSITIONS AND METHODS FOR TREATING SPINAL MUSCULAR ATROPHY	,	O'RIORDAN, CATHERINE R.
62561843	Not Issued		20	09/22/2017	VARIANT RNAi	,	O'RIORDAN, CATHERINE R.
08118054	Not Issued		162	09/08/1993	METHODS FOR PURIFYING CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR	WAX,ROBERT	O'RIORDAN, CATHERINE R.
08785107	5939536		250	01/21/1997	METHODS FOR PURIFYING CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATION	CARLSON,KAREN	O'RIORDAN, CATHERINE R.
09409803	6569426	20020034498	250	09/30/1999	TRESYL-MONOMETHOXYPOLYETHYLENE GLYCOL-MODIFIED VIRUSES HAVING VIRAL INFECTIVITY	NAFF,DAVID	O'RIORDAN, CATHERINE R.
09426680	6287857	0	250	10/25/1999	NUCLEIC ACID DELIVERY VEHICLES	SANDALS,WILLIAM	O'RIORDAN, CATHERINE R.
60002967	Not Issued		159	08/30/1995	PURIFICATION OF ADENOVIRUS AND AAV	,	O'RIORDAN, CATHERINE R.
60107471	Not Issued		159	11/06/1998	NUCLEIC ACID DELIVERY VEHICLES	,	O'RIORDAN, CATHERINE R.
60110899	Not Issued		159	12/04/1998	DRY POWDER COMPLEXES FOR GENE DELIVERY	,	O'RIORDAN, CATHERINE R.

Inventor Search Completed: No Records to Display.

Search Another: Inventor **Last Name** O'RIORDAN **First Name** CATHERINE Search

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EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L22	455	ultracentrifugation.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:47
L23	89174	viral.clm. or virus.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:47
L24	130	22 and 23	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:47
L25	46	24 and (sedimentation)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:48
L26	4797	sedimentation.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:48
L27	6	25 and 26	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:48
L28	2	("6989264"|"8137948").PN.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:49

EAST Search History (Interference)

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	953	genzyme.as.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:44
L2	25	(oriordan with catherine).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L3	25	(oriordan with catherine).in. or (burnham with brenda).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L4	1	(oriordan with catherine).in. and (burnham with brenda).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L5	1	(oriordan).in. and (burnham).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L6	1750	(oriordan).in. or (burnham).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L7	18	6 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L8	1	5 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L9	1	4 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L10	1	5 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L11	18	3 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
L12	18	2 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L13	455	ultracentrifugation.clm.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L14	89174	viral.clm. or virus.clm.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L15	130	13 and 14	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L16	2	15 and 1	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L17	1	15 and 2	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L18	1	15 and 3	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L19	1	15 and 4	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L20	1	15 and 5	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
L21	1	15 and 6	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Field

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15/544,498
	Filing Date	January 19, 2016 (Intl)
	First Named Inventor	Catherine R. O'RIORDAN
	Art Unit	1648
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	15979-20103.00

U.S. PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-6,989,264-B2		01-24-2006	Atkinson et al.	
	2.	US-8,137,948-B2		05-21-2003	Qu et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Country Code[2]	Foreign Document Number[3]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T[5]
	3.	WO-2010/148143-A1			12-23-2010	Genzyme Corporation		

NON-PATENT LITERATURE DOCUMENTS

Examiner 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.	T[5]
	4.	BERKOWITZ, S.A. et al. (March 1, 2007, e-pub. December 20, 2006). "Monitoring the Homogeneity of Adenovirus Preparations (A Gene Therapy Delivery System) Using Analytical Ultracentrifugation," *Anal. Biochem.* 362(1):16-37.	
	5.	BO, H. et al. (January 25, 2015, e-pub. November 26, 2014). "Chromatographic Purification of Adenoviral Vectors on Anion-Exchange Resins," *Eur. J. Pharm. Sci.* 67:119-125.	
	6.	BURNHAM, B. et al. (December 1, 2015, e-pub. October 15, 2015). "Analytical Ultracentrifugation as an Approach to Characterize Recombinant Adeno-Associated Viral Vectors," *Hum. Gene Ther. Methods* 26(6):228-242.	
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pa-1840115

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	8.	COLE, J.L. et al. (2008). "Analytical Ultracentrifugation: Sedimentation Velocity and Sedimentation Equilibrium," Methods *Cell Biol.* 84:143-179.	
	9.	FURST, A. (May 1997). "The XL-I Analytical Ultracentrifuge with Rayleigh Interference Optics," *Eur. Biophys. J.* 35(5-6):307-310.	
	10.	GOINS, W.F. et al. (2014). "Engineering HSV-1 Vectors for Gene Therapy," Chapter 5 *in* Herpes Simplex Virus: Methods in Molecular Biology, Springer Science+Business Media, New York, 1144:63-79.	
	11.	International Search Report mailed December 4, 2016, for PCT Application No. PCT/US2016/013947, filed January 19, 2016, 3 pages.	
	12.	KAPRANOV, P. et al. (January 2012, e-pub. August 29, 2011). "Native Molecular State of Adeno-Associated Viral Vectors Revealed by Single-Molecule Sequencing," *Hum. Gene Ther.* 23(1):46-55.	
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	17.	SOMMER, J.M. et al. (January 2003). "Quantification of Adeno-Associated Virus Particles and Empty Capsids by Optical Density Measurement," *Mol Ther.* 7(1):122-128.	
	18.	VAN TRICHT, E. (July 1, 2013). "Analytical Sciences Virus Particle Characterization Techniques to Quantify Virus Particle Aggregation and Integrity," MSc Chemistry, Literature Thesis, University Van Amsterdam, 51 pages.	

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	15979-20103.00

	19.	WRIGHT, J.F. (March 3, 2014). "Product-Related Impurities in Clinical-Grade Recombinant AAV Vectors: Characterization and Risk Assessment," *Biomedicines* 2(1):80-97.	
	20.	XIAO, X. et al. (March 1998). "Production of High-Titer Recombinant Adeno-Associated Virus Vectors in the Absence of Helper Adenovirus," *J. Virol.* 72(3):2224-2232.	
	21.	YANG, X. et al. (February 1, 2008). Determination of Particle Heterogeneity and Stability of Recombinant Adenovirus by Analytical Ultracentrifugation in CsCl Gradients," *J. Pharm. Sci.* 97(2):746-763.	

EXAMINER SIGNATURE

Examiner Signature	/RACHEL B GILL/	Date Considered	08/10/2018

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	First Named Inventor	Catherine R. O'RIORDAN
	Art Unit	1648
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	15979-20103.00

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (1).

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☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2018-04-06
Name/Print	Brian Donahue	Registration Number	58,206

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Application Serial No: 15/544,498

Attorney Docket No: 15979-20103.00
Customer Number: 89300
Date of Electronic Notification: 08/21/2018

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/544,498	07/18/2017	Catherine R. O'RIORDAN	15979-20103.00	2986

89300 7590 11/02/2018

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
GILL, RACHEL B

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
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PTOL-90A (Rev. 04/07)

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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR/ PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
15/544,498	07/18/2017	O'RIORDAN et al.	15979-20103.00

	EXAMINER	
Sanofi/Genzyme c/o Morrison & Foerster LLP 755 Page Mill Road Palo Alto, CA 94304	RACHEL B GILL	
	ART UNIT	PAPER
	1648	20181029

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Commissioner for Patents

Applicant's representative contacted Examiner regarding Cole reference (Cole JL. Characterization of human cytomegalovirus protease dimerization by analytical centrifugation. Biochemistry. 1996 Dec 3;35(48):15601-10. PubMed PMID: 8952514.) A PTO-892 is attached, along with a copy of said reference.

/RACHEL B GILL/
Primary Examiner, Art Unit 1648

PTO-90C (Rev.04-03)

Applicant-Initiated Interview Summary	**Application No.** 15/544,498	**Applicant(s)** O'RIORDAN et al.	
	Examiner RACHEL B GILL	**Art Unit** 1648	**AIA Status** Yes

All participants (applicant, applicants representative, PTO personnel):

(1) RACHEL B. GILL-examiner. (3) _____.

(2) Jessica Jarecki-agent. (4) _____.

Date of Interview: 29 October 2018.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☐ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: _____.

Identification of prior art discussed: Cole 1996.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant's representative contacted Examiner regarding Cole reference (Cole JL. Characterization of human cytomegalovirus protease dimerization by analytical centrifugation. Biochemistry. 1996 Dec 3;35(48):15601-10. PubMed PMID: 8952514.) Reference was incorrectly listed as "applicant cited prior art" in outstanding Office action, and applicant is requesting a copy of said reference. A PTO-892 is attached, along with a copy of said reference..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/RACHEL B GILL/ Primary Examiner, Art Unit 1648	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

Notice of References Cited	Application/Control No. 15/544,498	Applicant(s)/Patent Under Reexamination O'RIORDAN et al.	
	Examiner RACHEL B GILL	Art Unit 1648	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A					
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Cole JL. Characterization of human cytomegalovirus protease dimerization by analytical centrifugation. Biochemistry. 1996 Dec 3; 35(48):15601-10. PubMed PMID: 8952514.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/544,498	07/18/2017	Catherine R. O'RIORDAN	15979-20103.00	2986

89300 7590 11/16/2018

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
GILL, RACHEL B

ART UNIT	PAPER NUMBER
1648	

NOTIFICATION DATE	DELIVERY MODE
11/16/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Office Action Summary	**Application No.** 15/544,498	**Applicant(s)** O'RIORDAN et al.	
	Examiner RACHEL B GILL	**Art Unit** 1648	**AIA Status** Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 06 April 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-10,12-18,23,30-37,42-44,48,58,61,67-68 and 80 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-10,12-18,23,30-37,42-44,48,58,61,67-68 and 80 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 18 July 2017 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☑ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Disposition of Claims

Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 67-68, and 80 are pending.

Examiner's Note

All paragraph numbers (¶) throughout this office action, unless otherwise noted, are from the US PGPub of this application 2018/0180525 A1, Published 06/28/2018.

The Examiner would like to note that interview requests are welcomed by the Office, but a request for an in-person interview at the Alexandria Office location must be made in a timely fashion prior to the interview, as the Examiner is not currently located at the Alexandria Office. Applicant is encouraged to utilize the new web-based Automated Interview Request (AIR) tool for submitting interview requests; more information can be found at https://www.uspto.gov/patent/laws-and-regulations/interview-practice.

Optional Authorization to Initiate Electronic Communications

The Applicant's representative may wish to consider supplying a written authorization in response to this Office action to correspond with the Examiner via electronic mail (e-mail). This authorization is **optional** on the part of the Applicant's representative, but it should be noted that the Examiner may not initiate nor respond to communications via electronic mail unless and until Applicant's representatives authorize such communications in writing within the official record of the patent application. A sample authorization is available at MPEP § 502.03, part II.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on 04/06/2018 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

Applicant Initiated Interview

Applicant's representative contacted the Office to clarify the Cole references cited herein, as there appeared to be discrepancies between the cited reference and the paragraph/page numbers within the rejection. The rejections have been updated and/or maintained from the previous Office action mailed 08/10/2018 as noted herein.

Claim Objections

(**Objection maintained**.) Claim 1 is objected to because of the following informalities: "recombinant viral" is repeated in lines 9-10. Appropriate correction is required.

Claim Rejections - 35 USC § 112(b); Second Paragraph

> The following is a quotation of 35 U.S.C. 112(b):
> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.
>
> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

(**Rejection maintained**.) Claims 12 and 15 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

The claims provide for variant genomes which may be truncated genomes, aggregates, DNA impurities, or recombinants. However, it is not clear how the recombinant variant genomes differ from the "true" recombinant genomes from which the claims depend.

For at least these reasons, claims 12 and 15 are rejected on the grounds of being indefinite.

Claim Rejections - 35 USC § 102

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.

(**Rejection citation updated**.) Claims 1, 18, 23, 30-37, 58, and 61 are rejected under 35 U.S.C. 102(a)(1) as being anticipated by Cole et. al. (Cole JL, et. al. *Methods Cell Biol.* 2008;84:143-79.; APPLICANT-CITED PRIOR ART; hereafter "Cole".) NB: the rejection was previously and incorrectly noted as being anticipated by "Cole JL. *Biochemistry.* 1996 Dec 3;35(48):15601-10", which is the incorrect citation for the Cole reference.

The claims are interpreted as being drawn to the following:

Claim 1 is drawn to a method of characterizing a preparation of recombinant viral particles comprising the steps of:

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle.

Further limitations of the method of claim 1 are wherein the sedimentation of recombinant viral particles is monitored by absorbance or interference (claim 18); wherein the preparation is an aqueous solution (claim 23); wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant viral particles (claim 30);

wherein the C(s) values are determined by an algorithm that comprises Lamm equation solutions (claim 31); wherein the algorithm is the SEDFIT algorithm (claim 32); wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge (claim 33); wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell (claim 34); wherein sedimentation is monitored until recombinant viral particles sediment to the bottom of an ultracentrifuge velocity cell (claim 35); wherein sedimentation is monitored until the recombinant viral particles with the lowest density sediments and clears an optical window (claim 36); wherein at least 30 scans are used to monitor sedimentation of recombinant viral particles (claim 37); wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds (claim 58); and wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm and/or at about 4°C to about 20 °C (claim 61).

The Prior Art

Cole teaches that Analytical ultracentrifugation (AUC) is a versatile and powerful method for the quantitative analysis of macromolecules in solution (abstract) and can be utilized to determine the size and shape of macromolecules in solution, such as viruses (p. 2, ¶2). Note that "recombinant viral particle" is not specifically defined in the specification (¶[0058] appears to be specifically drawn to "recombinant AAV particles"). Furthermore, "recombinant viral vectors" or "recombinant viruses" appear to be defined in the specification as a virus comprising one or more heterologous sequences, such as nucleic acid not of viral origin. However, homologous recombination is a natural process amongst many viruses, such as herpesviruses, and recombine to incorporate genetic material not of herpesvirus origin, i.e. genetic information from other viruses, bacteria, or host cells. Therefore, "recombinant" is an adjective that is not interpreted to provide any meaningful limitation to the term "virus" or "viral particle", and "recombinant viral particles" and "recombinant viruses" are interpreted to be equivalent to "viral particles" and "viruses".

Cole teaches subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals (p. 3, ¶1), plotting the differential sedimentation coefficient distribution value (C(s)) (p. 5, ¶3) versus the sedimentation coefficient (s) (p. 3, ¶1) in Svedberg units (S) (p. 3, ¶3-4; p. 4, ¶3; p. 15, ¶2; See "B. Data Analysis" starting on p. 15, Table 4) and integrating the area under each peak in the C(s)

distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle (abstract; p. 4, ¶1; p. 5, ¶1; pp. 15-16, ¶ bridging pages; See "B. Data Analysis" starting on p. 15, esp. ¶ bridging pp. 15-16.) Therefore, Cole anticipates the limitations of **instant claim 1**.

Cole teaches the sedimentation from aqueous solutions is monitored by absorbance, interference, or fluorescence (abstract; **instant claims 18, 23**). Cole teaches adjusting the optics to account for the solvent component interference in the sample solution (p. 7, ¶2) and the use of reference solutions in absorbance optics for AUC (p. 7, ¶5; **instant claim 30**). Cole teaches that the C(s) values are determined by an algorithm that comprises Lamm equation solutions (p. 3, ¶2-4; p. 16, ¶3; **instant claim 31**); wherein the algorithm is the SEDFIT algorithm (p. 12, ¶3; p. 16, ¶ 3; p. 17, ¶2; **instant claim 32**). Cole teaches that sedimentation is monitored according to the sedimentation coefficient of the sample, and should take a boundary at least 2 hours to sediment the full length of a cell to the bottom of a sector (p. 15; ¶2; **instant claim 33**). Cole teaches optimizing solvents according to their compatibility with the sample cell and with optical systems (p. 2, ¶2; **instant claim 34**). Cole describes the instrumentation, and details the rotors, sample holders, and optical systems, including:

"To view the sample, the analytical rotor has holes through it to hold sample containers commonly called cells. Each cell contains a centerpiece, with chambers (called channels) to hold the liquid samples. The centerpiece, in turn, is sealed between windows to permit the passage of light through the channels, thus allowing the cell contents to be viewed."

The instrumentation described would allow monitoring of the sedimentation of particles within the AUC through an optical window (p. 6, ¶2; **instant claim 35-**36). Cole describes the "scans" performed to view the sedimentation (p. 6, ¶3) and states when observing the system using absorbance readings it is best to scan at least 30-40 times (p. 15, ¶2; **instant claim 37**) and depending on the scanning method used, the scans can be performed once every second up to once every 300 seconds (Table 2; **instant claim 58**.) In analyzing the data using SEDFIT, regularization procedures can be performed to produce a smoother distribution function (p, 16, ¶3). Cole describes that depending on what you want to know about your sample and how you are scanning the sample will alter the velocity of the rotor, and that depending on the S (Svedberg units) of the sample, you will use a velocity of anywhere from 10,000-55,000 RPM

(Table 3), and with absorbance scanning (especially involving multi-wavelength scanning), one may wish to use a lower speed (Table 3; p. 19, ¶3-4, 6,000-12,000 RPM, p. 7, ¶4) and that larger biomolecules may aggregate or degrade, so one can detect the loss of material by measuring the OD at low speed (3,000 RPM; pp. 12-13, ¶ bridging pages; **instant claim 61**). Cole describes that with some AUCs it is difficult to use the machine with a temperature below 4 °C and that most experiments are performed at about 20 °C (pp. 14-15, ¶ bridging pages; **instant claim 61**).

For at least these reasons, Cole teaches the limitations of claims 1, 18, 23, 30-37, 58, and 61 and anticipates the instant invention.

(**Rejection maintained**.) Claims 1-10, 12-18, 23, 30-36, 61, and 67-68 are rejected under 35 U.S.C. 102(a)(1) as being anticipated by van Tricht (van Tricht E. "Virus Particle Characterization: Techniques to Quantify Virus Particle Aggregation and Integrity." Literature Thesis, MSc Chemistry, Analytical Sciences. Univ. of Amsterdam, 07/2013; APPLICANT-CITED PRIOR ART; hereafter "van Tricht".)

The claims are interpreted as being drawn to the following:

The scope of claims 1, 18, 23, 30-37, 58, and 61 has been set forth *supra*.

Further limitations of the method of claim 1 are wherein the recombinant viral particle is a recombinant adeno-associated viral (AAV) particle, a recombinant adenovirus particle, a recombinant lentivirus particle or a recombinant herpes simplex viral (HSV) particle (claim 67).

Claims 2-10 and 12-17 are variations on instant claim 1 that assess, via AUC, vector genome integrity of viral particles (claims 2-3), empty capsid presence (claims, 4, 10, 12), empty capsid amounts (claims 5-6), relative amount of variant viral particles (claims 6-7), relative amount of intact viral particles with full genomes (claim 8), removal/lack of empty capsids (claim 9), and/or determination/monitoring of heterogeneity of viral particles (claims 13-17), and/or monitoring production of viral particles (claim 68).

The Prior Art

Van Tricht discloses the assessment of the suitability of several techniques for the characterization of virus particle (in particular adenovirus, see e.g. p. 25, ¶2) aggregation or integrity, including AUC under boundary conditions. Van Tricht teaches subjecting the aqueous preparation (p. 31,

¶2; **instant claim 23**) to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant viral particles is monitored at time intervals using absorbance (pp. 19-20, ¶ bridging pages, Fig. 4; **instant claim 18**) and compared to a reference (Fig. 4; **instant claim 30**), plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient (s) in Svedberg units (S) and integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant viral particle (Fig. 7.) Therefore, Van Tricht anticipates the limitations of **instant claim 1**. Data analysis using SEDFIT/Lamb is shown just as in present application (see e.g. Fig.7, pp. 23-4 shows monomer v. dimer distribution; **instant claims 31-32**). Van Tricht teaches sedimentation is monitored within the cell until the recombinant viral particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge (pp. 19-20, Fig. 4; **instant claims 33-36**).

Van Tricht teaches AUC of adenovirus particles (p. 25, starting at ¶2) wherein said particles were run on a CsCl gradient to have optimal separation between complete (mature) and incomplete (empty capsid) particles based on density differences. Said experiment was performed using absorption monitoring of the CsCl gradient run for 18 hours at 4 deg C at 40,000 RPM (p. 25, **instant claims 61, 67**). Van Tricht teaches separation showed 2 different types of empty capsids and 4 other virus forms (p. 25), and the methods not only assess empty and mature capsids, but also aggregates of virus and proteins. Van Tricht teaches that empty capsids and aggregates should be monitored and removed from manufacturing processes since they can have toxic side effects when used in vaccine preparations (pp. 11-12, pp.13-14, ¶ bridging pages; p. 6, ¶2.) As set forth *supra*, claims 2-10 and 12-17 are variations on instant claim 1 that assess, via AUC, vector genome integrity of viral particles (claims 2-3), empty capsid presence (claims, 4, 10, 12), empty capsid amounts (claims 5-6), relative amount of variant viral particles (claims 6-7), relative amount of intact viral particles with full genomes (claim 8), removal/lack of empty capsids (claim 9), determination/monitoring of heterogeneity of viral particles (claims 13-17) and/or monitoring production of viral particles (claim 68). The methods of Van Tricht therefore teach the methods not only of **instant claim 1**, but also **instant claims 2-10, 12-17, and 68**.

Van Tricht therefore teaches the limitations of instant claims 1-10, 12-18, 23, 30-36, 61, and 67-68, and anticipates the instant invention.

Claim Rejections - 35 USC § 103

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

(**Rejection maintained**.) Claims 37, 42-44, 48, and 58 are rejected under 35 U.S.C. 103 as being unpatentable over van Tricht as applied to claims 1-10, 12-18, 23, 30-36, 61, and 67-68 above, and further in view of Cole (*supra*) and Shuck (Schuck P. *Biophys J.* 2000 Mar;78(3):1606-19; APPLICANT-CITED PRIOR ART; hereafter "Shuck".)

The claims are interpreted as being drawn to the following:

The scope of claims 1-10, 12-18, 23, 30-37, 58, 61, and 67-68 have been set forth *supra*.

Further limitations on the instant invention are wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68 (claim 42); wherein the regularization is a second derivative regularization (claim 43); wherein the regularization is Max entropy regularization (claim 44); wherein the following C(s) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software (claim 48); wherein the sedimentation of recombinant viral particles is monitored about every 10-60 seconds (claim 58);

The Prior Art

The teachings of van Tricht have been set forth *supra*. Van Tricht teaches AUC, but teaches more general methods and parameters for AUC. The teachings of Cole have been set forth *supra*, and render obvious the differences in teachings for van Tricht as far as AUC parameters such as temperature and rotor RPM. Optimization of conditions for the methods of van Tricht would be obvious to one of skill in the art, given the technical teachings of Cole and the further teachings of Shuck. While neither van Tricht nor Cole teach specific regularization methods of calculation, Shuck teaches maximum entropy regularization to represent a continuous size-distribution in analysis of macromolecules by sedimentation velocity ultracentrifugation (entire document; see abstract.) Shuck teaches optimization of sedimentation profiles, such as adjusting the maximum entropy regularization is adjusted to a probability of p = 0.68 with a F ratio (frictional ratio or F statistic) of 1.0 (Fig. 1). Shuck provides teachings and motivation to optimize the parameters of a sediment velocity-based AUC method to separate biomolecules, and the combined teachings of van Tricht, which teach AUC for adenovirus virions, Cole, which teaches the basic principles of AUC, and Shuck, which teach the more technical parameters of data analysis of AUC, would render obvious the limitations of **instant claims 42-44, 48, and 58**, especially through routine optimization one of skill would have to take in order to maximize the separation profiles of empty and mature viral particles.

It would have been obvious to one of ordinary skill in the art to modify the methods taught by van Tricht and Cole in order to optimize the AUC methods, thereby specifically identifying the data refining equations and methods to be used. One would have been motivated to do so, given the suggestion by Shuck that optimization of sedimentation profiles for separation of biomolecules using AUC is essential and routine. There would have been a reasonable expectation of success, given the knowledge as to

how to refine the data and protocol for biomolecules separated by AUC, as taught by Shuck. Thus the invention as a whole was clearly *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

(**Rejection maintained**.) Claim 80 is rejected under 35 U.S.C. 103 as being unpatentable over van Tricht as applied to claims 1-10, 12-18, 23, 30-36, 61, and 67-68 above, and further in view of Colosi (US 2009/0017542 A1, Pub. 01/15/2009, hereafter "Colosi".)

The claims are interpreted as being drawn to the following:

The scope of claims 1-10, 12-18, 23, 30-37, 58, 61, and 67-68 have been set forth *supra*.

Claim 80 is drawn to a method for preparing recombinant viral particles with reduced empty capsids and/or recombinant viral particles comprising variant genomes, the method comprising:

a) culturing host cells under conditions suitable for recombinant viral production, wherein the cells comprise:

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant viral particles;

c) isolating the recombinant viral particles produced by the host cell; and

d) analyzing the recombinant viral particles for the presence of empty capsids and/or recombinant viral particles with variant genomes by analytical ultracentrifugation by the method of claim 1.

The Prior Art

Van Tricht teaches the purification of adenoviruses using the method of claim 1, and teaches the feasibility of applying such purification methods to other systems, but is silent as to the purification of adeno-associated virus (AAV), a virus that, like adenovirus, is commonly studied for gene therapy and vaccine purposes.

Colosi teaches a method of producing recombinant AAV (rAAV) virions comprising:

(a) introducing an AAV vector into a suitable host cell;

(b) introducing a AAV helper function vector into the host cell; wherein said vector is comprising:

an AAV rep coding region;

an AAV cap coding region; and

a nucleotide sequence comprising a modified AAV p5 promoter region, such that the modified AAV p5 promoter region no longer functions in transcription initiation;

(c) expressing accessory functions in the host cell; and

(d) culturing the host cell to produce rAAV virions (reference claims 1-2, 5). Colosi teaches that with unlike previously described methods for producing rAAV, the AAV helper function vectors generate high titer stocks of rAAV but do not produce any detectable wild-type AAV (¶[0029]).

Taking the teachings of van Tricht, which teach one of ordinary skill how to use AUC purification methods and teaches how such methods may be applied to other viruses, one of ordinary skill would be motivated to try said method on purifying AAV. Colosi teaches growing recombinant AAV (rAAV), but is silent as to the exact method of collecting the desired rAAV virions. One of skill in the art would turn to the methods available for virion purification, especially one that would allow one of skill to distinguish and separate empty capsids from other types of virions. The method of van Tricht would be an obvious choice to separate the virions grown by Colosi, as AAV is desired for use in gene therapy and vaccine compositions. Therefore, the limitations of **instant claim 80** would be obvious to one of ordinary skill in the art, given the teachings of Colosi and van Tricht.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RACHEL B GILL whose telephone number is (571)272-3129. The examiner can normally be reached on M to F 8:00 AM to 5:00 PM Eastern.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, JANET ANDRES can be reached on 571-272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RACHEL B GILL/
Primary Examiner, Art Unit 1648

Applicant-Initiated Interview Summary	**Application No.** 15/544,498	**Applicant(s)** O'RIORDAN et al.	
	Examiner RACHEL B GILL	**Art Unit** 1648	**AIA Status** Yes

All participants (applicant, applicants representative, PTO personnel):

(1) RACHEL B. GILL-examiner. (3) _____.

(2) Jessica Jarecki-agent. (4) _____.

Date of Interview: 14 November 2018.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☐ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Dr. Jarecki had contacted Ex. Gill regarding discrepancies with the Cole reference citation in the 102 and the rejection. The updated Office action has clarified the Cole rejection..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/RACHEL B GILL/ Primary Examiner, Art Unit 1648	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	Art Unit
	RACHEL B GILL	1648

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Google inventor, assignee, and keyword search	08/10/2018	RG
PALM inventor search (see attached)	08/10/2018	"
EAST inventor, assignee, and keyword search (see attached)	08/10/2018	"
PubMed inventor, assignee, and keyword search (see attached)	08/10/2018	"
Updated Google and PubMed searches	11/14/2018	RG

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/RACHEL B GILL/ Primary Examiner, Art Unit 1648	

Bibliographic Data

Application No: 15/544,498

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /RACHEL B GILL/ (Examiner's Signature) | (Initials)

Title: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
07/18/2017	424	1648	15979-20103.00
RULE			

APPLICANTS

Genzyme Corporation, Cambridge, MA, UNITED STATES

INVENTORS

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Brenda BURNHAM Hopkinton, MA, UNITED STATES

CONTINUING DATA

This application is a 371 of PCT/US2016/013947 01/19/2016

PCT/US2016/013947 has PRO of 62105714 01/20/2015

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

03/22/2018

STATE OR COUNTRY

UNITED STATES

ADDRESS

Sanofi/Genzyme c/o Morrison & Foerster LLP

755 Page Mill Road

Palo Alto, CA 94304

UNITED STATES

FILING FEE RECEIVED

$1,880

PTO/SB/06 (09-11)
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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.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/544,498	Filing Date 07/18/2017	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $80 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $420 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT	02/15/2019	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 35	Minus	** 35	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 9	Minus	*** 9	= 0		x $460 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/JAMES W TUNSTALL/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No.: 15979-20103.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patents Application of:
Catherine R. O'RIORDAN et al.

Application No.: 15/544,498

Confirmation No.: 2986

Filed: January 19, 2016 (Int'l)

Art Unit: 1648

For: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Examiner: R. Gill

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. 1.111

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

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the non-final Office Action dated November 16, 2018, for which a response is due on February 16, 2019. Accordingly, this response is timely filed. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 11 of this paper.

pa-1864427

Electronic Acknowledgement Receipt	
EFS ID:	35166735
Application Number:	15544498
International Application Number:	
Confirmation Number:	2986
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20103.00
Receipt Date:	15-FEB-2019
Filing Date:	18-JUL-2017
Time Stamp:	16:41:47
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		ROA.pdf	138991 a93b715080d52388c5a33b958d891e5aa8f51622	yes	16

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Applicant Arguments/Remarks Made in an Amendment	11	16
	Claims	2	10
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Warnings:			
Information:			
	Total Files Size (in bytes):	138991	

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.

REMARKS

Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 67-68, and 80 were pending in the present application. By virtue of this response, claim 67 has been canceled, and claims 1-10, 12-18, 30, 33, 35-37, 58, 68, and 80 have been amended. Accordingly, claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 68, and 80 are currently under consideration.

Support for the claim amendments can be found throughout the specification, for example paragraphs [0026], [0087], and [0166]-[0169]. No new matter is believed to be added by way of these amendments.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Statement of Substance of the Interview

Applicant thanks Examiner Rachel Gill for the courtesy of conducting the telephonic interview with Applicant representatives Jessica Jarecki on October 29, 2018 and November 14, 2018.

During the telephone interviews, Applicant's representative discussed with the Examiner the discrepancies between the cited reference and the paragraph and page numbers within the rejection. The reissuance of the Office Action by the Examiner in view of the discrepancies is greatly appreciated.

Applicants believe this response fulfills the duty to respond to the Examiner' s Interview Summary dated November 2, 2018 and November 16, 2018.

Rejections under 35 U.S.C. §112

Claims 12 and 15 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. Applicant respectfully traverses.

The Examiner alleges that "it is not clear how the recombinant variant genomes differ from the 'true' recombinant genomes from which the claims depend." Solely for purposes of expediting prosecution and without acquiescing to the rejection, claims 12 and 15 have been amended to recite that "the variant genomes are truncated AAV genomes, aggregates, recombinants and/or DNA impurities compared to the intact AAV genome." Accordingly, claims 12 and 15 are definite and Applicant respectfully requests that the rejection be withdrawn.

Rejections under 35 U.S.C. §102

Claims 1, 18, 23, 30-37, 58, and 61 are rejected under 35 U.S.C. § 102(a)(1) as allegedly being anticipated by Cole (Cole JL, *et al.*, *Methods Cell Biol.* 2008; 84:143-79, hereinafter "Cole"). Applicant respectfully traverses.

Solely for purposes of expediting prosecution and without acquiescing to the rejection, independent claim 1 has been amended to recite that the viral particles are recombinant adeno-associated viral (AAV) particles. Cole is silent regarding recombinant AAV particles. Accordingly, claim 1 and claims dependent therefrom are novel over Cole. Applicant respectfully requests that the rejection be withdrawn.

Additionally, Applicant respectfully disagrees with the Examiner's interpretation of the term "recombinant viral particle" as equivalent to "viral particles." As discussed above, claim 1 has been amended to recite "recombinant adeno-associated viral (AAV) particles." As discussed in paragraphs [0058] and [0059], a recombinant AAV (rAAV) particle "refers to a viral particle

composed of at least one AAV capsid protein and an encapsidated rAAV vector genome," and a recombinant AAV vector "refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of AAV origin) that are flanked by at least one AAV inverted terminal repeat sequences (ITR)." According to paragraph [0063], "'Heterologous' means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide)." The Examiner has provided no evidence that naturally occurring AAV particles express heterologous polynucleotides.

Claims 1-10, 12-18, 23, 30-36, 61, and 67-68 are rejected under 35 U.S.C. § 102(a)(1) as allegedly being anticipated by van Tricht (van Tricht E. "Virus Particle Characterization: Techniques to Quantify Virus Particle Aggregation and Integrity." Literature Thesis, MSc Chemistry, Analytical Sciences. Univ. of Amsterdam, 07/2013; hereinafter "van Tricht".) Applicant respectfully traverses.

Solely for purposes of expediting prosecution and without acquiescing to the rejection, independent claims 1, 2, 4-8, and 13 have been amended to recite that the viral particles are recombinant AAV particles. van Tricht is silent regarding recombinant AAV particles. Accordingly, claims 1, 2, 4-8, and 13 and claims dependent therefrom are novel over van Tricht. Applicant respectfully requests that the rejection be withdrawn.

Rejections under 35 U.S.C. §103

Claims 37, 42-44, 48, and 58 are rejected under 35 U.S.C. § 103 as allegedly being unpatentable over van Tricht, and further in view of Cole and Schuck (Schuck P. *Biophys J.* 2000 Mar;78(3):1606-19; hereinafter "Schuck"). Applicant respectfully traverses.

As discussed above, van Tricht and Cole are completely silent regarding recombinant AAV particles, much less using analytical ultracentrifugation (AUC) as claimed to measure the relative amount of capsid particles comprising variant recombinant AAV genomes or empty AAV capsid particles in a preparation of recombinant AAV particles. Schuck does not remedy the deficiencies of van Tricht and Cole, as Schuck is also silent regarding recombinant AAV particles.

Further, one of skill in the art would not have predicted with a reasonable expectation of success that methods described for a virus such as adenovirus could be applied to recombinant AAV particles. Adenoviruses and AAV are distinctly different viruses in a number of ways, but they especially differ in terms of size as adenoviruses are about 500-1000% larger than AAV in terms of both genome size and actual particle size. In addition, adenovirus and AAV are different in terms of capsid complexity as adenovirus capsids are made up of hexons, pentons, fibers and knobs whereas AAV capsids are made up of three VP proteins with greatly overlapping amino acid sequences. One of skill in the art would not have assumed that the use of AUC would have allowed for the characterization of variant recombinant viral genomes or empty viral capsid particles in a preparation of recombinant AAV particles, much less with the sensitivity, precision, and accuracy discussed below.

The current application is based on the inventors' surprising finding that AUC could be applied to preparations of recombinant AAV particles to identify variant recombinant viral genomes or empty viral capsid particles impurities with incredible sensitivity, precision, and accuracy. Briefly, the inventors analyzed several preparations of recombinant AAV with known sizes by AUC and generated a standard curve. Measurements of preparations of recombinant AAV were so precise that the R^2 value of the standard curve was 0.9978. The measurements were also so accurate that they were able to distinguish recombinant viral particles with a less than 200 nucleotides difference in size, see Example 5 and FIG. 8. In addition, the use of AUC on preparations of recombinant AAV was shown to be superior to Southern Blot as Southern Blot was unable to detect impurities that were detected by AUC, see Example 6.

Given the silence of cited references regarding the use of AUC for recombinant AAV particles, the lack of a reasonable expectation of success in using such a technique, and the

surprising results discussed above, claims 37, 42-44, 48, and 58 are not obvious in view of van Tricht, Cole, and Schuck. Applicant respectfully requests that the rejection be withdrawn.

Claim 80 is rejected under 35 U.S.C. § 103 as allegedly being unpatentable over van Tricht, and further in view of Colosi (US 2009/0017542 A1, hereinafter "Colosi"). Applicant respectfully traverses.

As discussed above, van Tricht is completely silent regarding recombinant AAV particles, much less using AUC as claimed to measure the relative amount of capsid particles comprising variant recombinant AAV genomes or empty AAV capsid particles in a preparation of recombinant AAV particles. Colosi does not rectify the deficiencies of van Tricht. Colosi described methods of making recombinant AAV particles, but Colosi is silent regarding any method of determining recombinant AAV particle impurities, much less AUC. Given the significant differences between the adenoviruses disclosed in van Tricht as discussed above, one of skill in the art would not have predicted with a reasonable expectation of success that methods described for a virus such as adenovirus could be applied to recombinant AAV particles. One of skill in the art certainly would not have expected the sensitivity, precision, and accuracy discussed above.

Given the silence of cited references regarding the use of AUC for recombinant AAV particles, the lack of a reasonable expectation of success in using such a technique, and the surprising results discussed above, claim 80 is not obvious in view of van Tricht and Colosi. Applicant respectfully requests that the rejection be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20103.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: February 15, 2019

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
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AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (Currently amended): A method of characterizing a preparation of recombinant adeno-associated viral (AAV) particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), and

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each peak, wherein each peak represents a species of recombinant AAV ~~viral recombinant viral~~ particle.

Claim 2 (Currently amended): A method to assess vector genome integrity of recombinant AAV [[viral]] particles in a preparation of recombinant AAV [[viral]] particles comprising

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value C(s) versus the sedimentation coefficient in Svedberg units (S), and

c) identifying species of recombinant AAV [[viral]] particles in the preparation by presence of peaks on the plot corresponding to an S value, wherein the genome size of a particular species of recombinant AAV ~~viral recombinant viral~~ particles is calculated by comparing the S value of the species to a standard curve generated by S values of recombinant AAV [[viral]] particles comprising encapsidated AAV [[viral]] genomes of known nucleotide sizes.

Claim 3 (Currently amended): The method of claim 2 further comprising integrating the area under each peak in the C(s) distribution to determine the relative concentration of each species of recombinant AAV ~~viral recombinant viral~~ particles.

Claim 4 (Currently amended): A method to determine the presence of empty capsids or capsid particles comprising variant sized recombinant AAV [[viral]] genomes in a preparation of recombinant AAV [[viral]] particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals, and

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant AAV [[viral]] genomes indicates that presence of capsid particles comprising variant sized genomes and/or empty capsids.

Claim 5 (Currently amended): A method of measuring the relative amount empty capsids in a preparation of recombinant AAV [[viral]] particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each species of recombinant AAV [[viral]] particles, and

d) comparing the amount of recombinant AAV [[viral]] particles having an S value corresponding to empty capsid particles to the amount of recombinant AAV [[viral]] particles having an S value corresponding to recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes or the total amount of recombinant AAV [[viral]] particles in the preparation.

Claim 6 (Currently amended): A method of measuring the relative amount of capsid particles comprising variant recombinant AAV [[viral]] genomes or empty AAV [[viral]] capsid particles in a preparation of recombinant AAV [[viral]] particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each species of recombinant AAV [[viral]] particles,

d) comparing the amount of recombinant AAV [[viral]] particles having an S values that do not correspond to recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes to the amount of recombinant AAV [[viral]] particles having an S value that corresponds to recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes or to the total amount of recombinant AAV [[viral]] particles in the preparation.

Claim 7 (Currently amended): A method of measuring the relative amount of capsid particles comprising variant recombinant AAV [[viral]] genomes in a preparation of recombinant AAV [[viral]] particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each species of recombinant AAV [[viral]] particles,

d) comparing the amount of recombinant AAV [[viral]] particles having an S values that do not correspond to recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes or empty capsid particles to the total amount of recombinant AAV [[viral]] particles in the preparation.

Claim 8 (Currently amended): A method of measuring the relative amount of recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes in a preparation of recombinant AAV [[viral]] particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S),

c) integrating the area under each peak in the C(s) distribution to determine the relative concentration of each species of recombinant AAV [[viral]] particles,

d) comparing the amount of recombinant AAV [[viral]] particles having an S values corresponding to recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes to the amount of recombinant AAV [[viral]] particles having an S value corresponding to empty capsid particles, to capsid particles comprising variant recombinant AAV [[viral]] genomes, and/or to the total amount of recombinant AAV [[viral]] particles in the preparation.

Claim 9 (Currently amended): A method of monitoring the removal of empty capsids and/or capsid particles comprising variant recombinant AAV [[viral]] genomes during the purification of a preparation of recombinant AAV [[viral]] particles, the method comprising removing a sample of the recombinant AAV [[viral]] particles from the preparation following one or more steps in the purification process and analyzing the sample for the relative amount of empty capsids and/or capsid particles comprising variant recombinant AAV [[viral]] genomes according to the method of claim 5, wherein a decrease in the relative amount of empty capsids and/or capsids comprising variant genomes to full capsids indicates removal of empty capsids from the preparation of recombinant AAV [[viral]] particles.

Claim 10 (Currently amended): The method of claim 4, wherein the presence of a peak that corresponds to the S value of empty capsid particles indicates the presence of empty capsid particles; or the presence of one or more peaks other than the peak for full capsid particles

comprising intact recombinant AAV [[viral]] genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes.

Claim 11 (Canceled)

Claim 12 (Currently amended): The method of claim 10, wherein the presence of one or more peaks other than the peak for full capsid particles comprising intact recombinant AAV [[viral]] genomes or empty capsid particles indicates that presence of capsid particles comprising variant sized genomes, and wherein the capsid particles comprising variant sized genomes comprises truncated genomes, aggregates, recombinants and/or DNA impurities compared to the intact recombinant AAV genome.

Claim 13 (Currently amended): A method of determining the heterogeneity of recombinant AAV [[viral]] particles in a preparation of recombinant AAV [[viral]] particles comprising the steps of

a) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions wherein the sedimentation of recombinant AAV [[viral]] particles is monitored at time intervals,

b) plotting the differential sedimentation coefficient distribution value (C(s)) versus the sedimentation coefficient in Svedberg units (S), wherein the presence of peaks in addition to the peak representing capsids comprising an intact AAV [[viral]] genome indicates heterogeneity of recombinant particles in the preparation.

Claim 14 (Currently amended): The method of claim 13, wherein the presence of additional peaks indicates the presence of empty capsid particles and/or recombinant AAV [[viral]] particles comprising variant genomes.

Claim 15 (Currently amended): The method of claim 14, wherein the variant genomes are truncated AAV [[viral]] genomes, aggregates, recombinants and/or DNA impurities compared to the intact recombinant AAV genome.

Claim 16 (Currently amended): The method of claim 13, further comprising integrating the area under each peak in the C(s) distribution to determine the relative concentration of each species of recombinant AAV [[viral]] particles.

Claim 17 (Currently amended): A method of monitoring the homogeneity of recombinant AAV [[viral]] particles during the purification of a preparation of recombinant AAV [[viral]] particles, the method comprising removing a sample of the recombinant AAV [[viral]] particles from the preparation following one or more steps in the purification process and determining the heterogeneity of recombinant AAV [[viral]] particles according to the method of claim 16, wherein an increase in the relative amount of recombinant AAV [[viral]] particles comprising intact viral genomes indicates an increase in the homogeneity of full AAV [[viral]] particles in the preparation of recombinant AAV [[viral]] particles.

Claim 18 (Currently amended): The method of claim 1, wherein the sedimentation of recombinant AAV [[viral]] particles is monitored by absorbance or interference.

Claims 19-22 (Canceled)

Claim 23 (Previously presented): The method of claim 1, wherein the preparation is an aqueous solution.

Claims 24-29 (Canceled)

Claim 30 (Currently amended): The method of claim 23, wherein the monitoring further comprises comparison to a reference sample, wherein the reference sample comprises the aqueous solution without recombinant AAV [[viral]] particles.

Claim 31 (Previously presented): The method of claim 1, wherein the C(s) values are determined by an algorithm that comprises Lamm equation solutions.

Claim 32 (Original): The method of claim 31, wherein the algorithm is the SEDFIT algorithm.

Claim 33 (Currently amended): The method of claim 1, wherein:
sedimentation is monitored until the recombinant AAV [[viral]] particles with the lowest density sediments to the bottom of a sector of an ultracentrifuge.

Claim 34 (Previously presented): The method of claim 1, wherein the ultracentrifugation utilizes an ultracentrifuge comprising an ultracentrifuge velocity cell.

Claim 35 (Currently amended): The method of claim 1, wherein sedimentation is monitored until recombinant AAV [[viral]] particles sediment to the bottom of an ultracentrifuge velocity cell.

Claim 36 (Currently amended): The method of claim 34, wherein sedimentation is monitored until the recombinant AAV [[viral]] particles with the lowest density sediments and clears an optical window.

Claim 37 (Currently amended): The method of claim 33, wherein at least 30 scans are used to monitor sedimentation of recombinant AAV [[viral]] particles.

Claims 38-41 (canceled)

Claim 42 (Previously presented): The method of claim 31, wherein a regularization is applied to a fitting level with a confidence level of F statistic of at least about 0.68.

Claim 43 (original): The method of claim 42, wherein the regularization is a second derivative regularization.

Claim 44 (original): The method of claim 42, wherein the regularization is Max entropy regularization.

Claims 45-47 (canceled)

Claim 48 (Previously presented): The method of claim 31, wherein the following C(S) parameters are held constant: resolution of about 200S to about 5000S, S min is about 1S to about 100S, S max is about 100S to about 5000S, and frictional ratio is about 1.0 or is left to float to a value determined by centrifugation software.

Claims 49-57 (canceled)

Claim 58 (Currently amended): The method of claim 1, wherein the sedimentation of recombinant AAV [[viral]] particles is monitored about every 10-60 seconds.

Claims 59-60 (Canceled)

Claim 61 (Previously presented): The method of claim 1, wherein the boundary sedimentation velocity is performed at about 3,000 rpm to about 20,000 rpm and/or at about 4°C to about 20 °C.

Claims 62-67 (Canceled)

Claim 68 (Currently amended): A method of evaluating a process for the production of recombinant AAV [[viral]] particles comprising the method of claim 1, wherein an increase in the relative amount of recombinant AAV [[viral]] particles comprising intact AAV [[viral]] genomes compared to the relative amount of empty capsid particles and/or recombinant AAV [[viral]] capsid particles with variant recombinant AAV [[viral]] genomes compared to a reference preparation of recombinant AAV [[viral]] particles indicates an improvement in the production of recombinant AAV [[viral]] particles.

Claims 69-79 (Canceled)

Claim 80 (Currently amended): A method for preparing recombinant AAV [[viral]] particles with reduced empty capsids and/or recombinant AAV [[viral]] particles comprising variant genomes, the method comprising

a) culturing host cells under conditions suitable for recombinant AAV [[viral]] production, wherein the cells comprise

i) nucleic acid encoding a heterologous transgene flanked by at least one AAV ITR,

ii) nucleic acid comprising AAV rep and cap coding regions, wherein the nucleic acid comprises a mutated p5 promoter wherein rep expression from the p5 promoter is reduced compared to a wild-type p5 promoter, and

iii) nucleic acid encoding AAV helper virus functions;

b) lysing the host cells to release recombinant AAV [[viral]] particles;

c) isolating the recombinant AAV [[viral]] particles produced by the host cell; and

d) analyzing the recombinant AAV [[viral]] particles for the presence of empty capsids and/or recombinant AAV [[viral]] particles with variant genomes by analytical ultracentrifugation by the method of claim 1.

Claims 81-95 (Canceled)

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NOTICE OF ALLOWANCE AND FEE(S) DUE

89300 7590 05/22/2019

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
GILL, RACHEL B	
ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 05/22/2019

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/544,498	07/18/2017	Catherine R. O'RIORDAN	15979-20103.00	2986

TITLE OF INVENTION: ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	08/22/2019

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

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PTOL-85 (Rev. 02/11)

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(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/544,498	07/18/2017	Catherine R. O'RIORDAN	15979-20103.00	2986

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nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	08/22/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
GILL, RACHEL B	1648	424-186100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required) ❑Advance Order - # of Copies _____

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5. **Change in Entity Status** (from status indicated above)

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❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

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Novartis Exhibit 1002, Page 554 of 591

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EXAMINER	
GILL, RACHEL B	
ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 05/22/2019

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/544,498	Applicant(s) O'RIORDAN et al.	
	Examiner RACHEL B GILL	Art Unit 1648	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to response filed on 02/15/2019.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are See Continuation Sheet . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file areply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/RACHEL B GILL/
Primary Examiner, Art Unit 1648

Continuation of 3. The allowed claim(s) is/are: 1-10,12-18,23,30-37,42-44,48,58,61,68 and 80

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Disposition of Claims

Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 67-68, and 80 were pending. Claims 11, 19-22, 24-29, 38-41, 45-47, 49-57, 59-60, 62-67, 69-79, and 81-95 have been cancelled. Amendments to claims 1-10, 12-18, 30, 33, 35-37, 58, 68, and 80 are acknowledged and entered. Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 68, and 80 will be examined on their merits.

Examiner's Note

All paragraph numbers (¶) throughout this office action, unless otherwise noted, are from the US PGPub of this application 2018/0180525 A1, Published 06/28/2018.

The Examiner would like to note that interview requests are welcomed by the Office, but a request for an in-person interview at the Alexandria Office location must be made in a timely fashion prior to the interview, as the Examiner is not currently located at the Alexandria Office. Applicant is encouraged to utilize the new web-based Automated Interview Request (AIR) tool for submitting interview requests; more information can be found at https://www.uspto.gov/patent/laws-and-regulations/interview-practice.

Response to Arguments

Applicant's arguments filed 02/15/2019 regarding the previous Office action dated 11/16/2018 have been fully considered. If they have been found to be persuasive, the objection/rejection has been withdrawn below. Likewise, if a rejection/objection has not been recited, said rejection/objection has been withdrawn. If the arguments have not been found to be persuasive, or if there are arguments presented over art that has been utilized in withdrawn rejections but utilized in new rejections, the arguments will be addressed fully with the objection/rejection below.

Claim Objections

(**Objection withdrawn**.) The objection to Claim 1 because of typographical informalities is **withdrawn** in light of the amendments to the claims.

Claim Rejections - 35 USC § 112(b); Second Paragraph

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

(**Rejection withdrawn**.) The rejection of Claims 12 and 15 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention, is **withdrawn** in light of the amendments to the claims.

Claim Rejections - 35 USC § 102

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

(**Rejection withdrawn**.) The rejection of Claims 1, 18, 23, 30-37, 58, and 61 under 35 U.S.C. 102(a)(1) as being anticipated by Cole et. al. (Cole JL, et. al. *Methods Cell Biol*. 2008;84:143-79.; APPLICANT-CITED PRIOR ART; hereafter "Cole") is **withdrawn** in light of the amendments to the claims.

(**Rejection withdrawn**.) The rejection of Claims 1-10, 12-18, 23, 30-36, 61, and 67-68 under 35 U.S.C. 102(a)(1) as being anticipated by van Tricht (van Tricht E. "Virus Particle Characterization: Techniques to Quantify Virus Particle Aggregation and Integrity." Literature Thesis, MSc Chemistry, Analytical Sciences. Univ. of Amsterdam, 07/2013; APPLICANT-CITED PRIOR ART; hereafter "van Tricht") is **withdrawn** in light of the amendments to the claims.

Claim Rejections - 35 USC § 103

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

(**Rejection withdrawn**.) The rejection of Claims 37, 42-44, 48, and 58 under 35 U.S.C. 103 as being unpatentable over van Tricht as applied to claims 1-10, 12-18, 23, 30-36, 61, and 67-68 above, and further in view of Cole (*supra*) and Shuck (Schuck P. *Biophys J*. 2000 Mar;78(3):1606-19; APPLICANT-CITED PRIOR ART; hereafter "Shuck") is **withdrawn** in light of the amendments to the claims and applicant arguments.

(**Rejection withdrawn**.) The rejection of Claim 80 under 35 U.S.C. 103 as being unpatentable over van Tricht as applied to claims 1-10, 12-18, 23, 30-36, 61, and 67-68 above, and further in view of Colosi (US 2009/0017542 A1, Pub. 01/15/2009, hereafter "Colosi") is **withdrawn** in light of the amendments to the claims and applicant arguments.

Allowable Subject Matter

Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 68, and 80 are allowed.

The following is an examiner's statement of reasons for allowance: Analytical ultracentrifugation (AUC) was well-described in the art as a means to separate biological molecules, including viruses. While it would be obvious to apply AUC to separation of AAV particles and routine to optimize said parameters, AUC was not noted as having been applied to AAV separation routinely, and the general methods of separating AAV in the art were not very accurate or specific, especially when it came to variation within the AAV genome or the AAV subtype, and separation of empty AAV particles, which were known to cause unwanted effects when AAV preparations were used *in vivo*. Applicants have surprisingly found parameters for AUC that allow for high levels of separation of AAV particles, including accurate separation of AAV subtypes and empty particles. The closest prior art is that of record, which teaches general methods of AUC, but the argument that the results were surprising and/or unexpected (see p. 4 of "Remarks" starting at ¶2) was determined to be convincing after a thorough search of the art.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Claims 1-10, 12-18, 23, 30-37, 42-44, 48, 58, 61, 68, and 80 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RACHEL B GILL whose telephone number is (571)272-3129. The examiner can normally be reached on M to F 8:00 AM to 5:00 PM Eastern.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, JANET ANDRES can be reached on 571-272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RACHEL B GILL/
Primary Examiner, Art Unit 1648

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	Art Unit
	RACHEL B GILL	1648

CPC								
Symbol							Type	Version
G01N	/	15	/	042			F	2013-01-01
C12N	/	7	/	00			I	2013-01-01
G01N	/	1	/	405			I	2013-01-01
G01N	/	1	/	4077			I	2013-01-01
G01N	/	2001	/	4083			A	2013-01-01
C12N	/	2710	/	10351			A	2013-01-01
C12N	/	2750	/	14151			A	2013-01-01
G01N	/	2015	/	045			A	2013-01-01

CPC Combination Sets									
Symbol							Type	Set	Ranking	Version
	/		/							

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	34	
/RACHEL B GILL/ Primary Examiner, Art Unit 1648	16 May 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20190515

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	Art Unit
	RACHEL B GILL	1648

INTERNATIONAL CLASSIFICATION				
CLAIMED				
G01N15/04	/	15	/	04
C12N7/00	/	7	/	00
G01N1/40	/	1	/	40
CPCONLY	/	NLY	/	

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	34	
/RACHEL B GILL/ Primary Examiner, Art Unit 1648	16 May 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20190515

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	**Art Unit**
	RACHEL B GILL	1648

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	34	
/RACHEL B GILL/ Primary Examiner, Art Unit 1648	16 May 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office Part of Paper No.: 20190515

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	Art Unit
	RACHEL B GILL	1648

CPC - Searched*		
Symbol	**Date**	**Examiner**
C07K2317/92 OR C07K14/005 OR A61K39/12 OR C12N15/86 OR C12N2750/14143 OR C12N15/864 OR C12N2750/14152 OR G01N33/68 OR G01N2001/4083 OR C12Q1/70	05/16/2019	RG
G01N15/042 or G01N1/4077 or G01N1/405 or C12N7/00 or G01N2015/045 or C12N2750/14151 or C12N2710/10351 or G01N2001/4083 or C12N2750/14151	05/16/2019	"

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	**Date**	**Examiner**
Google inventor, assignee, and keyword search	08/10/2018	RG
PALM inventor search (see attached)	08/10/2018	"
EAST inventor, assignee, and keyword search (see attached)	08/10/2018	"
PubMed inventor, assignee, and keyword search (see attached)	08/10/2018	"
Updated Google and PubMed searches	11/14/2018	RG
Updated Google, PALM, EAST, and PubMed searches	05/16/2019	RG

/RACHEL B GILL/ Primary Examiner, Art Unit 1648	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/544,498	O'RIORDAN et al.
	Examiner	Art Unit
	RACHEL B GILL	1648

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	EAST inventor, assignee, keyword, and CPC interference search (see EAST search results)	05/16/2019	RG

/RACHEL B GILL/ Primary Examiner, Art Unit 1648	

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	480	ultracentrifugation.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L2	93749	viral.clm. or virus.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L3	137	L1 and L2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L4	48	L3 and (sedimentation)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L5	5018	sedimentation.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L6	8	L4 and L5	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L7	2	("6989264"|"8137948").PN.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32

EAST Search History (Prior Art)

L29	63192	"analytical ultracentrifugation" or "AUC"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L30	2374	"analytical ultracentrifugation".clm. or "AUC".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:32
L31	68558	"adeno-associated virus" or "AAV"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:33
L32	4138	"adeno-associated virus".clm. or "AAV".clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:33
L33	1	32 and 30	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:33
L34	195	32 and 29	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:33
L35	2314	"analytical ultracentrifugation"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:33

EAST Search History (Prior Art)

L36	12	35 and 32	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:33
L45	416	("adeno-associated virus" or "AAV") and ("analytical ultracentrifugation")	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:35
L46	73405	(G01N15/042 or G01N1/4077 or G01N1/405 or C12N7/00 or G01N2015/045 or C12N2750/14151 or C12N2710/10351 or G01N2001/4083).cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:40
L49	73405	(G01N15/042 or G01N1/4077 or G01N1/405 or C12N7/00 or G01N2015/045 or C12N2750/14151 or C12N2710/10351 or G01N2001/4083 or C12N2750/14151).cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:43
L50	165	45 AND ((C07K2317/92 OR C07K14/005 OR A61K39/12 OR C12N15/86 OR C12N2750/14143 OR C12N15/864 OR C12N2750/14152 OR G01N33/68 OR G01N2001/4083 OR C12Q1/70).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:44
L51	232575	(C07K2317/92 OR C07K14/005 OR A61K39/12 OR C12N15/86 OR C12N2750/14143 OR C12N15/864 OR C12N2750/14152 OR G01N33/68 OR G01N2001/4083 OR C12Q1/70).CPC.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/05/16 08:44
S1	455	ultracentrifugation.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:47

EAST Search History (Prior Art)

S2	89174	viral.clm. or virus.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:47
S3	130	S1 and S2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:47
S4	46	S3 and (sedimentation)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:48
S5	4797	sedimentation.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:48
S6	6	S4 and S5	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/08/10 08:48
S7	2	("6989264"|"8137948").PN.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:49

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L8	983	genzyme.as.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L9	27	(oriordan with catherine).in.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L10	27	(oriordan with catherine).in. or (burnham with brenda).in.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32

EAST Search History (Interference)

L11	1	(oriordan with catherine).in. and (burnham with brenda).in.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L12	1	(oriordan).in. and (burnham).in.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L13	1781	(oriordan).in. or (burnham).in.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L14	18	L13 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L15	1	L12 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L16	1	L11 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L17	1	L12 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L18	18	L10 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L19	18	L9 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L20	480	ultracentrifugation.clm.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L21	93749	viral.clm. or virus.clm.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L22	137	L20 and L21	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L23	2	L22 and L8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L24	1	L22 and L9	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L25	1	L22 and L10	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L26	1	L22 and L11	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L27	1	L22 and L12	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L28	1	L22 and L13	US-PGPUB; USPAT	OR	ON	2019/05/16 08:32
L37	2374	"analytical ultracentrifugation".clm. or "AUC".clm.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:34
L38	4138	"adeno-associated virus".clm. or "AAV".clm.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:34
L39	2	37 and 8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:35
L40	1	37 and 9	US-PGPUB; USPAT	OR	ON	2019/05/16 08:35
L41	1	37 and 10	US-PGPUB; USPAT	OR	ON	2019/05/16 08:35

EAST Search History (Interference)

L42	1	37 and 11	US-PGPUB; USPAT	OR	ON	2019/05/16 08:35
L43	1	37 and 12	US-PGPUB; USPAT	OR	ON	2019/05/16 08:35
L44	1	37 and 38	US-PGPUB; USPAT	OR	ON	2019/05/16 08:35
L47	16590	(G01N15/042 or G01N1/4077 or G01N1/405 or C12N7/00 or G01N2015/045 or C12N2750/14151 or C12N2710/10351 or G01N2001/4083).cpc.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:40
L48	16590	(G01N15/042 or G01N1/4077 or G01N1/405 or C12N7/00 or G01N2015/045 or C12N2750/14151 or C12N2710/10351 or G01N2001/4083 or C12N2750/14151).cpc.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:43
L52	46560	(C07K2317/92 OR C07K14/005 OR A61K39/12 OR C12N15/86 OR C12N2750/14143 OR C12N15/864 OR C12N2750/14152 OR G01N33/68 OR G01N2001/4083 OR C12Q1/70).CPC.	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L53	10453	48 and 52	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L54	7	53 and 9	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L55	40	53 and 8	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L56	10359	53 and "7"	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L57	7	53 and 10	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L58	1	53 and 11	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L59	1	53 and 12	US-PGPUB; USPAT	OR	ON	2019/05/16 08:44
L60	6	38 and 54	US-PGPUB; USPAT	OR	ON	2019/05/16 08:45
L61	37	38 and 55	US-PGPUB; USPAT	OR	ON	2019/05/16 08:45
L62	1	37 and 60	US-PGPUB; USPAT	OR	ON	2019/05/16 08:45
L63	1	37 and 61	US-PGPUB; USPAT	OR	ON	2019/05/16 08:45
S8	953	genzyme.as.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:44
S9	25	(oriordan with catherine).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S10	25	(oriordan with catherine).in. or (burnham with brenda).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45

EAST Search History (Interference)

S11	1	(oriordan with catherine).in. and (burnham with brenda).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S12	1	(oriordan).in. and (burnham).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S13	1750	(oriordan).in. or (burnham).in.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S14	18	S13 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S15	1	S12 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S16	1	S11 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S17	1	S12 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S18	18	S10 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:45
S19	18	S9 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S20	455	ultracentrifugation.clm.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S21	89174	viral.clm. or virus.clm.	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S22	130	S20 and S21	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S23	2	S22 and S8	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S24	1	S22 and S9	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S25	1	S22 and S10	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S26	1	S22 and S11	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S27	1	S22 and S12	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46
S28	1	S22 and S13	US-PGPUB; USPAT	OR	ON	2018/08/10 08:46

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Day : Wednesday
Date: 5/15/2019
Time: 18:04:56

Inventor Name Search Result

Your Search was:

Last Name = BURNHAM
First Name = BRENDA

Application#	Patent#	PG Pub#	Status	Date Filed	Title	Examiner Name	Inventor Name
15544498	Not Issued	20180180525	71	07/18/2017	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	GILL,RACHEL	BURNHAM, BRENDA
62105714	Not Issued		159	01/20/2015	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	,	BURNHAM, BRENDA

Inventor Search Completed: No Records to Display.

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#39	Add	**Similar articles** for **PubMed** (Select **29349097**)	93	18:13:12
#37	Add	**Similar articles** for **PubMed** (Select **28166648**)	143	18:13:06
#35	Add	**Similar articles** for **PubMed** (Select **26958574**)	85	18:13:00
#3	Add	Search **(O'riordan c[au]) AND burnham b[au]**	4	18:12:52
#33	Add	**Similar articles** for **PubMed** (Select **3094412**)	70	18:12:45
#31	Add	**Similar articles** for **PubMed** (Select **9330762**)	174	18:12:25
#29	Add	**Similar articles** for **PubMed** (Select **2156205**)	87	18:12:02
#27	Add	**Similar articles** for **PubMed** (Select **13668550**)	95	18:11:44
#25	Add	Search **(svedberg units) AND (virus or "viral particle")**	11	18:11:33
#24	Add	Search **svedberg units**	53	18:11:19
#23	Add	**Cited In** for **PubMed** (Select **26414997**)	9	18:11:04
#21	Add	**Similar articles** for **PubMed** (Select **27196374**)	101	18:10:56
#19	Add	**Similar articles** for **PubMed** (Select **17223062**)	173	18:10:34
#17	Add	**Similar articles** for **PubMed** (Select **14507723**)	94	18:10:29
#15	Add	**Similar articles** for **PubMed** (Select **11806949**)	134	18:10:16
#13	Add	**Similar articles** for **PubMed** (Select **10048319**)	88	18:10:07
#11	Add	**Similar articles** for **PubMed** (Select **7212420**)	125	18:10:01
#9	Add	**Similar articles** for **PubMed** (Select **840648**)	101	18:09:55
#7	Add	Search **(((analytical ultracentrifugation) AND boundary sedimentation)) AND (virus or viral)**	8	18:09:50
#6	Add	Search **(analytical ultracentrifugation) AND boundary sedimentation**	61	18:09:37

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#5	Add	Search **boundary sedimentation**	315	18:09:34
#4	Add	Search **analytical ultracentrifugation**	4208	18:09:11
#2	Add	Search **burnham b[au]**	83	18:08:51
#1	Add	Search **O'riordan c[au]**	60	18:08:43

Novartis Exhibit 1002, Page 577 of 591

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Day : Wednesday
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Time: 18:04:09

Inventor Name Search Result

Your Search was:

Last Name = O'RIORDAN
First Name = CATHERINE

Application#	Patent#	PG Pub#	Status	Date Filed	Title	Examiner Name	Inventor Name
09029483	Not Issued		157	02/08/1998	VIRAL VECTOR CONSTRUCTS	KAUSHAL,SUMESH	O'RIORDAN, CATHERINE
11934148	Not Issued	20090069261	120	11/02/2007	GENE THERAPY FOR SPINAL CORD DISORDERS	WEHBE,ANNE MARIE	O'RIORDAN, CATHERINE
12328267	9034836	20100267812	150	12/04/2008	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
12417910	9890394	20090286857	150	04/03/2009	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
14692469	Not Issued	20160067312	120	04/21/2015	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
15127757	Not Issued	20170173183	98	09/20/2016	GENE THERAPY FOR RETINITIS PIGMENTOSA	BURKHART,MICHAEL	O'RIORDAN, CATHERINE
15549825	Not Issued	20180023082	71	08/09/2017	VARIANT RNAi	CHONG,KIMBERLY	O'RIORDAN, CATHERINE
15858303	Not Issued	20180230490	41	12/29/2017	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	CHEN,SHIN LIN	O'RIORDAN, CATHERINE
16325653	Not Issued		19	01/01/0001	METHODS FOR DETECTING AAV	,	O'RIORDAN, CATHERINE
60135092	Not Issued		159	02/08/1998	VIRAL VECTOR CONSTRUCTS	NOT,DEFINED	O'RIORDAN, CATHERINE
60827977	Not Issued		159	10/03/2006	GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS AND OTHER SPINAL CORD DISORDERS	,	O'RIORDAN, CATHERINE
61045977	Not Issued		159	04/15/2008	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RdCVF)	,	O'RIORDAN, CATHERINE
61969027	Not Issued		159	03/21/2014	GENE THERAPY FOR RETINITIS PIGMENTOSA	,	O'RIORDAN, CATHERINE
62105714	Not Issued		159	01/20/2015	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	,	O'RIORDAN, CATHERINE
62114576	Not Issued		159	02/10/2015	NOVEL NUCLEIC ACIDS FOR IMPROVED GENE DELIVERY	,	O'RIORDAN, CATHERINE
62114578	Not Issued		159	02/10/2015	VARIANT RNAi	,	O'RIORDAN, CATHERINE
62375814	Not Issued		159	08/15/2016	METHODS FOR DETECTING AAV	,	O'RIORDAN, CATHERINE
62561934	Not Issued		159	09/22/2017	COMPOSITIONS AND METHODS OF TREATING HUNTINGTON'S DISEASE	,	O'RIORDAN, CATHERINE
09604349	Not Issued		161	06/27/2000	CHROMATOGRAPHIC PURIFICATION OF ADENOVIRUS	SALIMI,ALI	O'RIORDAN, CATHERINE E.
10476604	7915026	20040152183	250	03/02/2004	PURIFICATION OF ADENOVIRUS AND AAV	SALIMI,ALI	O'RIORDAN, CATHERINE E.
11179865	7579181	20050244942	250	07/12/2005	PURIFICATION OF ADENOVIRUS AND AAV	MOSHER,MARY	O'RIORDAN, CATHERINE E.
12504073	Not Issued	20100279385	161	07/16/2009	PURIFICATION OF ADENOVIRUS AND AAV	MOSHER,MARY	O'RIORDAN, CATHERINE E.
09011828	6143548		150	06/29/1998	CHROMATOGRAPHIC PURIFICATION OF ADENOVIRUS AND AAV	SALIMI,ALI	O'RIORDAN, CATHERINE E.
60071674	Not Issued		159	01/16/1998	ADENOVIRAL VECTORS WITH MODIFIED CAPSID PROTEINS	,	O'RIORDAN, CATHERINE E.
60027653	Not Issued		159	08/24/1998	CATIONIC COMPLEXES OF POLYMER-MODIFIED ADENOVIRUS	,	O'RIORDAN, CATHERINE E.
60183296	Not Issued		159	02/17/2000	Adenoviral system	,	O'RIORDAN, CATHERINE R
09582509	Not Issued		161	06/26/2000	ADENOVIRAL VECTORS WITH MODIFIED CAPSID PROTEINS	GUZO,DAVID	O'RIORDAN, CATHERINE R.

09601022	Not Issued		161	07/24/2000	NUCLEIC ACID DELIVERY VEHICLES	GUZO,DAVID	O'RIORDAN, CATHERINE R.
10349630	Not Issued	20030180261	161	01/22/2003	Polymer-modified viruses	NAFF,DAVID	O'RIORDAN, CATHERINE R.
12936652	9567382	20110034546	150	10/06/2010	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)	BURKHART,MICHAEL	O'RIORDAN, CATHERINE R.
14828385	Not Issued	20160074474	30	02/08/2016	COMPOSITIONS AND METHODS FOR TREATING SPINAL MUSCULAR ATROPHY	SCHULTZ,JAMES	O'RIORDAN, CATHERINE R.
15397455	10035829	20170369541	150	01/03/2017	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)	BURKHART,MICHAEL	O'RIORDAN, CATHERINE R.
15544498	Not Issued	20180180525	71	07/18/2017	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES	GILL,RACHEL	O'RIORDAN, CATHERINE R.
16022410	Not Issued	20190055293	30	06/28/2018	METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)	BURKHART,MICHAEL	O'RIORDAN, CATHERINE R.
61818448	Not Issued		159	05/01/2013	COMPOSITIONS AND METHODS FOR TREATING SPINAL MUSCULAR ATROPHY	,	O'RIORDAN, CATHERINE R.
62561843	Not Issued		159	09/22/2017	VARIANT RNAi	,	O'RIORDAN, CATHERINE R.
08118054	Not Issued		162	09/08/1993	METHODS FOR PURIFYING CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR	WAX,ROBERT	O'RIORDAN, CATHERINE R.
08785107	5939536		250	01/21/1997	METHODS FOR PURIFYING CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATION	CARLSON,KAREN	O'RIORDAN, CATHERINE R.
09409803	6569426	20020034498	250	09/30/1999	TRESYL-MONOMETHOXYPOLYETHYLENE GLYCOL-MODIFIED VIRUSES HAVING VIRAL INFECTIVITY	NAFF,DAVID	O'RIORDAN, CATHERINE R.
09436680	6287857	0	250	10/25/1999	NUCLEIC ACID DELIVERY VEHICLES	SANDALS,WILLIAM	O'RIORDAN, CATHERINE R.
60002967	Not Issued		159	08/30/1995	PURIFICATION OF ADENOVIRUS AND AAV	,	O'RIORDAN, CATHERINE R.
60107471	Not Issued		159	11/06/1998	NUCLEIC ACID DELIVERY VEHICLES	,	O'RIORDAN, CATHERINE R.
60110892	Not Issued		159	12/04/1998	DRY POWDER COMPLEXES FOR GENE DELIVERY	,	O'RIORDAN, CATHERINE R.

Inventor Search Completed: No Records to Display.

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Document Description: Issue Fee Payment (PTO-85B)

Issue Fee Transmittal Form

Application Number	Filing Date	First Named Inventor	Atty. Docket No.	Confirmation No.
15544498	18-Jul-2017	Catherine O'RIORDAN	15979-20103.00	2986

TITLE OF INVENTION :

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

Entity Status	Application Type	Art Unit	Class - Subclass	EXAMINER
Regular Undiscounted	U.S. National Stage under 35 USC 371	1648	186100	RACHEL GILL

Issue Fee Due	Publication Due	Total Fee(s) Due	Date Due	Prev. Paid Fee
$1000	$0	$1000	22-Aug-2019	$0

1.Change of Correspondence Address and/or Indication Of Fee Address (37 CFR 1.33 & 1.363)

Current Correspondence Address:	Current Indicated Fee Address :
89300 Sanofi/Genzyme c/o Morrison & Foerster LLP 755 Page Mill Road Palo Alto CA 94304 UNITED STATES 650-813-5600 EOfficePA@mofo.com	
☐ Change of correspondence address requested, system generated AIA/122-EFS form attached	☐ Fee Address indication requested, system generated SB/47-EFS form attached

2.Entity Status

Change in Entity Status

○ Applicant certifying micro entity status; system generated Micro Entity certification form attached. See 37 CFR 1.29.
Note: Absent a valid certification of micro entity status, issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment. If this box is checked, you will be prompted to choose a micro entity status on the gross income basis (37 CFR 1.29(a)) or the institution of higher education basis (37 CFR 1.29(d)), and make the applicable certification online.

○ Applicant asserting small entity status. See 37 CFR 1.27.
Note: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

◉ Applicant changing to regular undiscounted fee status.
Note: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

Doc Code: IFEE PTOL/85B-EFS

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☐ Advance Order - # of copies _____	☒ If **in addition to** the payment of the issue fee amount submitted with this form, there are any discrepancies in any amount(s) due, the Director is authorized to charge any deficiency, or credit any overpayment, to Deposit Account Number 03-1952 . The **issue fee must be submitted** with this form. **If payment of the issue fee does not accompany this form, checking this box and providing a deposit account number will NOT be effective to satisfy full payment of the fee(s) due.**

4.Firm and/or Attorney Names To Be Printed

NOTE: If no name is listed, no name will be printed
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1. MORRISON & FOERSTER LLP
2.
3.

5.Assignee Name(s) and Residence Data To Be Printed

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

Name	City	State	Country	Category
Genzyme Corporation	Cambridge	MASSACHUSETTS	united states	corporation

6.Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via EFS-WEB on the date indicated below.

Signature	/Brian A. Donahue/	**Date**	08-21-2019
Name	Brian Arthur Donahue	**Registration Number**	58206

WEB IFEE 1.0

Electronic Patent Application Fee Transmittal

Application Number:	15544498
Filing Date:	18-Jul-2017
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Filer:	Brian Arthur Donahue/Aurelia Caparas
Attorney Docket Number:	15979-20103.00

Filed as Large Entity

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

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1000**

Electronic Acknowledgement Receipt	
EFS ID:	36933935
Application Number:	15544498
International Application Number:	
Confirmation Number:	2986
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20103.00
Receipt Date:	21-AUG-2019
Filing Date:	18-JUL-2017
Time Stamp:	15:52:21
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1000
RAM confirmation Number	E20198KF52165070
Deposit Account	
Authorized User	
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	Web85b.pdf	46359 6fab9683bac447b4406f8c9f07f08cafd075b389	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	32036 06a27162c8bdffba07040101a1c33822fcf256ba	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	78395		

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
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: IDS
Doc description: Information Disclosure Statement (IDS) Field

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15/544,498
	Filing Date	January 19, 2016 (Intl)
	First Named Inventor	Catherine R. O'RIORDAN
	Art Unit	1648
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	15979-20103.00

U.S. PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-6,989,264-B2		01-24-2006	Atkinson et al.	
	2.	US-8,137,948-B2		~~05-21-2003~~ 03-2012	Qu et al.	

Change(s) applied to document, /C.M./ 7/8/2019

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Country Code[2]	Foreign Document Number[3]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T[5]
	3.	WO-2010/148143-A1			12-23-2010	Genzyme Corporation		

NON-PATENT LITERATURE DOCUMENTS

Examiner 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.	T[5]
	4.	BERKOWITZ, S.A. et al. (March 1, 2007, e-pub. December 20, 2006). "Monitoring the Homogeneity of Adenovirus Preparations (A Gene Therapy Delivery System) Using Analytical Ultracentrifugation," *Anal. Biochem.* 362(1):16-37.	
	5.	BO, H. et al. (January 25, 2015, e-pub. November 26, 2014). "Chromatographic Purification of Adenoviral Vectors on Anion-Exchange Resins," *Eur. J. Pharm. Sci.* 67:119-125.	
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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.B.G/

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/544,498	10/01/2019	10429288	15979-20103.00	2986

89300 7590 09/11/2019

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Genzyme Corporation, Cambridge, MA;
Catherine R. O'RIORDAN, Bridgewater, NJ;
Brenda BURNHAM, Hopkinton, MA;

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IR103 (Rev. 10/09)

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Patent Number	10,429,288
Issue Date	October 1, 2019
Application Number	15/544,498
Filing Date	January 19, 2016 (Int'l)
First Named Inventor	Catherine R. O'RIORDAN
Attorney Docket Number	15979-20103.00

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Signature /Brian A. Donahue/

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Date January 22, 2020 | Telephone +1 (650) 813-5632

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Application Number:	15544498
International Application Number:	
Confirmation Number:	2986
Title of Invention:	ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES
First Named Inventor/Applicant Name:	Catherine R. O'RIORDAN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20103.00
Receipt Date:	22-JAN-2020
Filing Date:	18-JUL-2017
Time Stamp:	18:16:37
Application Type:	U.S. National Stage under 35 USC 371

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