IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

GENZYME CORPORATION, Plaintiff, v. SAREPTA THERAPEUTICS, INC., and SAREPTA THERAPEUTICS THREE, LLC. Defendants.	)	[redacted] **REDACTED PUBLIC VERSION** **FILED: June 4, 2025** C.A. No. 24-cv-00882-RGA JURY TRIAL DEMANDED

SECOND AMENDED COMPLAINT

Plaintiff Genzyme Corporation ("Genzyme"), by and through its undersigned attorneys, bring this action against Defendants Sarepta Therapeutics, Inc. ("Sarepta Therapeutics"), and Sarepta Therapeutics Three, LLC ("Sarepta Three") (together "Sarepta" or "Defendants").

NATURE OF ACTION

1. This is an action for infringement of United States Patent Nos. 9,051,542 (the "'542 Patent"), 7,704,721 (the "'721 Patent"), 12,031,894 (the "'894 Patent"), 12,013,326 (the "'326 Patent"), 11,698,377 (the "'377 Patent"), 12,123,880 (the "'880 Patent"), and 12,298,313 (the '313 Patent") (collectively, "the Patents-In-Suit") arising from Defendants' manufacture and sale of Elevidys® (delandistrogene moxeparvovec-rokl), a gene therapy for the treatment of a neuromuscular disease known as Duchenne muscular dystrophy ("DMD"). This action is based upon the Patent Laws of the United States, 35 U.S.C. §§ 100, *et seq.* True and correct copies of the '542 Patent and the '721 Patent are attached as Exhibit A and Exhibit B, respectively. True and correct copies of the '894 Patent, the '326 Patent, the '377 Patent, the '880 Patent, and the '313 Patent are attached as Exhibits O-S, respectively.

PARTIES

2. Plaintiff Genzyme is a corporation organized and existing under the laws of the Commonwealth of Massachusetts, having its principal place of business at 450 Water Street, Cambridge, MA 02141. Genzyme is the owner of the '542 Patent, the '721 Patent, the '894 Patent, the '326 Patent, the '377 Patent, the '880 Patent, and the '313 Patent.

3. Genzyme and its affiliates focus on the development of specialty treatments for debilitating diseases that are often difficult to diagnose and treat.

4. On information and belief, Defendant Sarepta Therapeutics is a company organized and existing under the laws of the State of Delaware, having its corporate offices and principal place of business at 215 First St., Cambridge, MA 02142. On information and belief, Sarepta Therapeutics has a registered agent for service of process, Corporation Service Company, 251 Little Falls Drive, Wilmington, DE 19808.

5. On information and belief, Defendant Sarepta Three is a corporation organized and existing under the laws of the State of Delaware, having its corporate offices and principal place of business at 215 First St., Cambridge, MA 02142. On information and belief, Sarepta Three has a registered agent for service of process, Corporation Service Company, 251 Little Falls Drive, Wilmington, DE 19808. On information and belief, Sarepta Therapeutics is the direct or indirect parent of Sarepta Three and has at all times directed and controlled the infringing actions of its subsidiary.

6. On information and belief, Sarepta Therapeutics is a biopharmaceutical company in the business of, among other activities, developing gene therapy products using adeno-associated virus ("AAV") technology to treat diseases, and Sarepta Three is engaged in the commercialization and/or manufacture of biopharmaceutical products in collaboration with Sarepta Therapeutics.

JURISDICTION AND VENUE

7. This is an action for patent infringement arising under the Patent Laws of the United States, 35 U.S.C. §§ 100 *et seq.*, including § 271(a). This Court has subject matter jurisdiction over this action under 28 U.S.C. §§ 1331 and 1338(a).

8. Venue is proper in this district pursuant to 28 U.S.C. § 1400(b) and/or 28 U.S.C. § 1391(b) and (c) for at least the reason that each Defendant resides in this district.

9. This Court has personal jurisdiction over Sarepta Therapeutics and Sarepta Three because they are incorporated in Delaware, knowingly transact business in Delaware, maintain a registered agent in Delaware, avail themselves of the rights and benefits of Delaware law, and, on information and belief, have engaged in, and made meaningful preparations to engage in, infringing conduct in Delaware.

10. On information and belief, each of the Defendants has established, and will continue to maintain, minimum contacts with this judicial district such that the exercise of jurisdiction over each of the Defendants would not offend traditional notions of fair play and substantial justice.

FACTUAL BACKGROUND

Gene Therapy Technology

11. It has long been recognized that certain diseases are caused by missing or defective genes, resulting in the inability of the body to produce key proteins. The result can be devastating, but the options for treating such genetic diseases have been limited. The radical approach taken by gene therapy is to attack the problem at the source—the patient's own genome—by providing a working copy of a defective or missing gene with what is referred to as a transgene. Gene therapy is at the cutting edge of medical technology, and the problems faced both in the delivery of transgenes and their manufacture are daunting.

12. Gene therapy can be performed by taking advantage of one of the body's age-old enemies, viruses, which have evolved to enter human cells. By removing part of the native viral DNA and substituting the DNA of the desired human transgene, a recombinant virus can be created that can enter cells and then deliver a desired human gene into a cell. At the time of the inventions of the Patents-In-Suit, it was known that in the right circumstances a modified adeno-associated virus ("AAV") could be used to achieve the delivery of a transgene. These genetically-engineered versions of the AAV are known as recombinant AAV ("rAAV") vectors.

13. Manufacturing rAAV-based therapeutics is a highly technical, multi-phase process involving rAAV vector production, which includes the creation of the vector genome, or genetic payload carrying the transgene, encapsidation of the vector genome in a protein shell called a capsid, followed by purification and formulation. A major concern during production is that the rAAV vector particles will become insoluble and aggregate into clusters of viral particles, which can result in production difficulties and loss of vector functionality. Low solubility and aggregation are problems thought to be attributable to the highly symmetrical nature of rAAV vector particles in conjunction with the stabilizing effect of complementary charged regions between neighboring particles in aggregates. Filtration can remove these aggregates during the purification process but at the cost of significantly reducing viral vector yields and thus increasing production costs.

14. Aggregation is particularly problematic with respect to formulations that are administered in ultraconcentrated, small volumes, as the high concentration levels promote aggregation. In such cases, aggregation can negatively impact the effectiveness of treatment, as well as increase the chance of an immune reaction following administration.

15. The inventions described in the '542 Patent and the '721 Patent are directed towards solving these problems. John Fraser Wright and Guang Qu, the inventors of the subject matter claimed in the '542 Patent and the '721 Patent, discovered that the use of certain high ionic strength solutions for preparing and storing rAAV vectors can prevent significant aggregation of virus particles at the concentrations needed for effective gene therapy. They invented rAAV formulations and related methods in which vector particles remain soluble when elevated ionic strengths are used during purification and for final vector formulation.

16. The process of creating an rAAV-based therapeutic involves the initial creation of the vector genome, followed by encapsidation of the vector genome in a protein shell called a capsid. Packaging the vector genome in the capsid is not a perfect process and it can result in a mixture of viral particles including capsids containing full, properly formed vector genomes, empty capsids that contain no vector genome, and partially filled capsids that contain fragmented or incomplete vector genomes.

17. Empty capsids and partially filled capsids are undesirable, and characterizing and purifying the preparations is a major difficulty in commercial manufacturing of rAAV therapies. In particular, it is critical to avoid introducing unwanted gene fragments and other impurities into patients. The Food and Drug Administration ("FDA") has identified these issues as raising important health concerns and has focused on the manufacturing process in evaluating which therapies will obtain marketing approval. *See, e.g.,* Cellular, Tissue, and Gene Therapies Advisory Committee, "Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy," FOOD AND DRUG ADMINISTRATION, Sept. 2-3, 2021, at §§ 2.3-2.4.1, 2.4.3 (last accessed May 13, 2025), https://www.nxgenvectorsolutions.com/wp-content/uploads/2024/01/FDA_CTGTAC-09.02.21-09.03.21-Meeting-Briefing-Document-FDA.pdf.

18. Determining the capsid and genome quality within an rAAV-based therapeutic is an important and necessary step in the manufacturing process. Prior to the inventions claimed in the '894 Patent and the '326 Patent, the DNA content of recombinant viral DNA vectors was typically determined by Southern blot analysis using a sequence specific probe. But Southern blot analysis is unable to detect fragmented genomes of unknown sequence. There was not a single assay that could distinguish full, properly-formed capsids from the undesired species—empty capsids and partially-filled capsids—on a quantitative basis.

19. The inventions described in the '894 Patent and the '326 Patent are directed towards solving that problem. Catherine R. O'Riordan and Brenda Burnham, the inventors of the subject matter claimed in the '894 Patent and the '326 Patent, developed techniques using analytical ultracentrifugation ("AUC") that allow for the detection and quantification of rAAV species, including full capsid particles, empty viral capsids with no rAAV genomes, and partially filled rAAV capsids, regardless of the nucleotide sequence of the recombinant viral genome or the serotype of the recombinant viral capsid. In so doing, the claimed AUC methods can be used to assess, quantitatively and qualitatively, an rAAV therapeutic for homogeneity, purity, and consistency of manufacturing. Thus, the '894 Patent and the '326 Patent represent a significant advancement to ensure consistency in the final gene therapy product.

20. rAAV viral capsid proteins, or "VPs," are the proteins that make up the viral shell encasing the transgene and are composed of three viral proteins: VP1, VP2 and VP3. These proteins are important to the viral infectivity and vector potency of rAAV therapies and, in turn, to enhancing safety and efficacy for patients. Post-translational modifications of these VPs, such as N-terminal acetylation, deamidation, glycosylation, and ubiquitination, can impact infectivity, potency, and heterogeneity of rAAV preparations, and in turn the safety and efficacy of rAAV

therapies comprising these VPs. Thus, accurate characterization of these VPs is critical, particularly as new rAAV therapies are brought into the clinic and to the market.

21. VP characterization was typically performed in the prior art through using sodium dodecyl sulfate polyacrylamide gel electrophoresis ("SDS-PAGE") analysis, followed by enzymatic digestion/gel separation and liquid chromatography/tandem mass spectrometry analysis ("LC/MS/MS"). This process resulted in a limited recovery of peptides after protein in-gel digestion and incomplete sequencing of the specific VPs, which led to potentially inaccurate results. Moreover, SDS-PAGE is not specific enough to differentiate every AAV serotype or post-translational modifications, including of the VPs.

22. The inventions described in the '880 Patent and the '313 Patent solved the problems that existed in the prior art. The inventors of the '880 Patent and the '313 Patent —Xiaoying Jin, Catherine O'Riordan, Lin Liu, and Kate Zhang—developed a method for analyzing VPs and AAV particles comprising such VPs. This method comprises denaturing AAV particles and subjecting the denatured particles to liquid chromatography/mass spectrometry ("LC/MS"). This method involves analysis of intact proteins, rather than proteins that have been digested or subjected to gel separation as in the prior art, to more accurately determine the masses of VPs in AAV particles. This method can be applied generally or specifically to more accurately determining posttranslational modifications of VPs, as described and claimed in the '880 Patent. This method can also be applied in the preparation of a pharmaceutical composition, as described and claimed in the '313 Patent.

23. Further, identification of the serotype of rAAV particles is important to the potency and selectivity of rAAV therapeutics, and in turn to improved safety and efficacy. Serotype identification was typically performed in the prior art by antibody-based enzyme-linked

immunosorbent assays or Western blotting, but these tests were generally not specific enough to differentiate between different rAAV serotypes. SDS-PAGE analysis followed by in gel digestion and LC/MS/MS analysis had also been used, but this extended process typically required several days and numerous steps, including digestion by multiple enzymes to obtain the full VP sequences.

24. The inventions described in the ’377 Patent include improved methods for analyzing the serotype of AAV particles. The improvements arise from the greater resolution of the claimed methods compared to the prior art. This improved resolution can differentiate between a wider range of serotypes, particularly those with high sequence homology and overlapping epitopes. This differentiation is particularly important given the increasing number of AAV serotypes and engineered capsids being developed for therapeutic applications. The inventors of the ’377 Patent—Xiaoying Jin, Catherine O’Riordan, Lin Liu, and Kate Zhang—developed methods for analyzing VPs and AAV particles comprising VPs. These methods comprise denaturing AAV particles and subjecting the denatured particles to LC/MS. These methods involve the analysis of intact proteins, rather than proteins that have been digested or subjected to gel separation as in the prior art, to more accurately determine the masses of VPs in AAV particles. These methods can be applied to more quickly and accurately determine the serotype of an AAV particle, as described and claimed in the ’377 Patent.

The Patents-in-Suit

The ’542 Patent

25. On June 9, 2015, the United States Patent & Trademark Office (“USPTO”) duly and legally issued the ’542 Patent, titled “Compositions and Methods to Prevent AAV Vector

Aggregation." The '542 Patent is assigned to Genzyme. A true and correct copy of the '542 Patent is attached as Exhibit A.

26. The claims of the '542 Patent are generally directed to the preparation of high ionic strength compositions for the storage of purified, rAAV vector particles in which the vector particles do not significantly aggregate. On June 15, 2023, Genzyme statutorily disclaimed claims 1 and 2 of the '542 Patent. Claims 3-6 of the '542 Patent expire on June 1, 2025.

The '721 Patent

27. On April 27, 2010, the USPTO duly and legally issued the '721 Patent, titled "Compositions and Methods to Prevent AAV Vector Aggregation." The '721 Patent is assigned to Genzyme. A true and correct copy of the '721 Patent is attached as Exhibit B.

28. The claims of the '721 Patent are generally directed to methods for the preparation of high ionic strength compositions for the storage of purified, rAAV vector particles in which the vector particles do not significantly aggregate. The claims of the '721 Patent expire on June 1, 2025.

The '894 Patent

29. On July 9, 2024, the USPTO duly and legally issued the '894 Patent, titled "Analytical Ultracentrifugation for Characterization of Recombinant Viral Particles." The '894 Patent is assigned to Genzyme. A true and correct copy of the '894 Patent is attached as Exhibit O.

30. The claims of the '894 Patent are generally directed to methods to quantify one or more species of individual variant viral particles comprising fragmented rAAV genomes in a heterogeneous mixture of viral particles using analytical ultracentrifugation. The claims of the '894 Patent expire on January 19, 2036.

The ’326 Patent

31. On June 18, 2024, the USPTO duly and legally issued the ’326 Patent, titled “Analytical Ultracentrifugation for Characterization of Recombinant Viral Particles.” The ’326 Patent is assigned to Genzyme. A true and correct copy of the ’326 Patent is attached as Exhibit P.

32. The claims of the ’326 Patent are generally directed to methods to determine the size of one or more fragmented genomes in a preparation of viral particles comprising rAAV vectors encapsidated into viral capsids and/or determine the molar concentrations of each species of individual viral particles in a heterogeneous mixture of viral particles comprising rAAV vectors encapsidated into viral capsids using analytical ultracentrifugation. The claims of the ’326 Patent expire on January 19, 2036.

The ’377 Patent

33. On July 11, 2023, the USPTO duly and legally issued the ’377 Patent, titled “Methods for Detecting AAV.” The ’377 Patent is assigned to Genzyme. A true and correct copy of the ’377 Patent is attached as Exhibit Q.

34. The claims of the ’377 Patent are generally directed to methods for determining the serotype of a denatured AAV particle by LC/MS intact protein analysis. The claims of the ’377 Patent expire on August 8, 2038.

The ’880 Patent

35. On October 22, 2024, the USPTO duly and legally issued the ’880 Patent, titled “Methods for Detecting AAV.” The ’880 Patent is assigned to Genzyme. A true and correct copy of the ’880 Patent is attached as Exhibit R.

36. The claims of the ’880 Patent are generally directed to methods for analyzing and determining post-translational modifications of denatured AAV particles by LC/MS intact protein analysis. The claims of the ’880 Patent expire on August 14, 2037.

The ’313 Patent

37. On May 13, 2025, the USPTO duly and legally issued the ’313 Patent, titled “Methods for Detecting AAV.” The ’880 Patent is assigned to Genzyme. A true and correct copy of the ’313 Patent is attached as Exhibit S.

38. The claims of the ’313 Patent are generally directed to methods for analyzing and determining post-translational modifications of denatured AAV particles by LC/MS intact protein analysis, and methods of preparing compositions comprising such AAV particles subjected to analysis and determination. The claims of the ’313 Patent expire on August 14, 2037.

Elevidys®

39. Sarepta Therapeutics is the holder of Biologics License Application (“BLA”) No. 125781 for Elevidys® (delandistrogene moxeparvovec-rokl) (also referred to as “SRP-9001”). Elevidys® is a one-time rAAV gene therapy product that is used to treat certain patients with DMD. A true and correct copy of the current Elevidys® package insert, dated August 2024, is attached as Exhibit C.

40. DMD is a form of muscular dystrophy caused by a mutation in the DMD gene that renders patients unable to produce a functional dystrophin protein. The disease typically strikes young boys around the age of four and leads to progressive muscle weakness. Patients with DMD experience various physical symptoms, including but not limited to, frequent falls, difficulty rising from a lying or sitting position, trouble running and jumping, waddling gait, and muscle pain and stiffness. By adolescence, many patients lose the ability to walk.

41. Elevidys® is designed to deliver a gene encoding a micro-dystrophin protein in the subject's muscle cells. On information and belief, the micro-dystrophin protein is a shortened, but functional, version of the dystrophin protein, comprising only selected domains and a fraction of the molecular weight of the dystrophin protein that is normally expressed in skeletal muscle cells. Elevidys® uses a non-replicating, rAAV vector of the serotype rh74 ("rAAVrh74") capsid to package and deliver a human micro-dystrophin transgene under the control of the MHCK7 promoter. *See* Exhibit C, § 11 Description.

42. On June 22, 2023, Sarepta obtained FDA accelerated approval to market Elevidys® for the treatment of ambulatory pediatric patients aged 4 through 5 years with DMD with a confirmed mutation in the DMD gene. *See* Exhibit D, Accelerated BLA Approval. On or about June 20, 2024, Sarepta subsequently obtained full FDA approval for an expanded indication for Elevidys® that significantly broadened the population of eligible patients to include DMD patients four years of age and older who are ambulatory and have a confirmed mutation in the DMD gene and accelerated approval for DMD patients four years of age and older who are non-ambulatory and have a confirmed mutation in the DMD gene. *See* Exhibit E, June 20, 2024, Supplemental Approval.

43. On information and belief, Sarepta has entered into agreements with Catalent, Inc. and/or Catalent Maryland, Inc. (collectively, "Catalent"), encompassing process development, clinical production and testing, and commercial manufacturing of Elevidys® for the U.S. market. *See* Sarepta Therapeutics, Inc., Annual Report (Form 10-K) p. 10 (Feb. 28, 2024), https://www.sec.gov/Archives/edgar/data/873303/000095017024022036/srpt-20231231.htm ("Sarepta 2024 Form 10-K"); Sarepta Therapeutics, Inc., Annual Report (Form 10-K) p. 9 (Feb. 28, 2025), https://www.sec.gov/Archives/edgar/data/873303/000095017025029973/srpt-

20241231.htm ("Sarepta 2025 Form 10-K"). On information and belief, Catalent manufactures the Elevidys® drug substance on behalf of Sarepta in Harmans, MD and the finished drug product in Baltimore, MD. *See* Exhibit F, § 3.2.A, Facilities Table. On information and belief, Elevidys® is manufactured by Catalent on Sarepta's behalf and under Sarepta's direction and control as the BLA holder. On information and belief, Catalent has been manufacturing Elevidys® on Sarepta's behalf and under Sarepta's direction and control, and Defendants have been marketing Elevidys® in the United States since obtaining FDA approval in 2023. *See* Exhibit D, Accelerated BLA Approval. On information and belief Defendants have formed a joint enterprise with third-party manufacturers and testing companies, such as Catalent, for the manufacture and sale of Elevidys®.

44. Detailed information about the manufacture of Elevidys®, including the release testing performed on Elevidys®, is contained in the Sarepta BLA, which was submitted to the FDA and is maintained by the FDA on a confidential basis.

45. As noted by Beckman Coulter, an analytical ultracentrifuge equipment manufacturer, analytical ultracentrifugation is acknowledged as the "gold standard" in the detection and quantification of AAV particles and "a valuable tool to analyze rAAV vectors notwithstanding the composition and length of the transgene or the viral serotype." Exhibit T.

46. Catalent also touts analytical ultracentrifugation as the "gold standard" method to detect and quantify capsids in a sample to ensure that patients are receiving a product containing the highest concentration of full rAAV capsids possible. *See* CATALENT BIOLOGICS, https://biologics.catalent.com/expert-content/gene-therapy/measuring-quality-attributes-for-gene-therapies-empty-vs-full-viral-vector-capsids/.

47. On information and belief, Defendants are required to verify the vector capsid identity, percent full capsid, capsid purity, and potency at least as part of the drug product release

specifications for Elevidys®. *See* Exhibit G, CBER CMC BLA Review Memo at § 3.2.P.5, Control of Drug Product. On information and belief, Defendants cannot sell Elevidys® without performing release testing. Identification of capsid purity includes identifying the uniformity of the capsid serotype such that it does not contain other serotypes that may exist through viral contamination.

48. In manufacturing Elevidys® drug product, on information and belief, Defendants quantify the viral particles in Elevidys® utilizing the claimed analytical ultracentrifugation methods of the '894 Patent and '326 Patent.

49. On information and belief, Defendants use the methods claimed in the '377 Patent to identify the rAAVrh74 capsid serotype of Elevidys® at various stages of manufacturing.

50. Sarepta is the current assignee of U.S. Patent Application US 2023/0204595 A1, entitled "Methods for analyzing AAV capsid proteins," to Daud et al., published June 29, 2023 (the "'595 Application"). *See* Exhibit U.

51. On information and belief, Defendants perform the methods described and claimed in the '595 Application as part of the release testing of commercial batches of Elevidys®. On information and belief, Defendants cannot sell Elevidys® without performing release testing. As described below, the methods practiced by Defendants infringe the '377 Patent and '880 Patent.

52. Example 4 of the '595 Application is entitled "Characterization of the VP1, VP2, and VP3 capsid proteins in an AAV particle." Example 4 of the '595 Application describes a method for analyzing VP1, VP2, and VP3 proteins in an rAAVrh74 capsid. Figure 3 and Table 6 of the '595 Application report the results of Example 4. According to the disclosures of the '595 Application, "Fig. 3 shows detection of post-translation modification of VP1, VP2, and VP3. Table 6 shows the intact mass analysis of AAV.rh74 capsid proteins."

53. The method set forth in Example 4 of the ’595 Application infringes at least one claim of each of the ’880 Patent, the ’377 Patent, and the ’313 Patent.

Sarepta’s Knowledge of the Patents-In-Suit

54. On July 26, 2024, Genzyme sent a notice letter to Sarepta advising Sarepta of Genzyme’s concerns that Sarepta was infringing the ’721 Patent, the ’542 Patent, the ’326 Patent, the ’894 Patent, and the ’377 Patent in its manufacture and sale of Elevidys®. Exhibit I.

55. On July 26, 2024, Genzyme filed a complaint for patent infringement in this matter (D.I. 1) alleging infringement of the ’542 Patent and the ’721 Patent by Sarepta by making, using, offering for sale, and/or selling Elevidys® in the United States in violation of 35 U.S.C. § 271(a), (b) and/or (c).

56. On October 31, 2024, Genzyme sent a second notice letter to Sarepta again advising Sarepta of Genzyme’s concerns that Sarepta was infringing the ’542 Patent, the ’721 Patent, the ’326 Patent, the ’894 Patent, the ’377 Patent, and the ’880 Patent in its manufacture and sale of Elevidys®. Exhibit J.

57. On May 1, 2025, Genzyme sent a third notice letter to Sarepta again advising Sarepta of Genzyme’s concerns that Sarepta was infringing the ’542 Patent, the ’721 Patent, the ’326 Patent, the ’894 Patent, the ’377 Patent, and the ’880 Patent, as well as U.S. Patent Application No. 19/013,863 (which issued as the ’313 Patent as set forth in the third notice letter) in its manufacture and sale of Elevidys®. Exhibit X.

58. On April 4, 2025, Sarepta served Objections and Responses to Genzyme’s First Set of Interrogatories (Nos. 1-10). In response to Interrogatory No. 6, Sarepta’s response states that based on its “investigation to-date and facts currently known to it,” Sarepta became aware of the

'542 patent on August 22, 2023 and became aware of the '721 patent on July 26, 2024. Exhibit K at 16-17.

59. On information and belief, because Sarepta was aware of the '542 patent at least as of August 22, 2023 and the '542 patent lists the '721 patent on its face, Sarepta also knew of and/or willfully disregarded the existence of the '721 patent at least as of August 22, 2023. On information and belief, Sarepta took deliberate actions to avoid learning of the existence of the '721 patent after gaining knowledge of the '542 patent.

60. On June 30, 2023, Sarepta filed a PCT International Search Report with the USPTO during prosecution of the '595 Application. *See* Exhibit V. This PCT search was purportedly completed by April 9, 2021. This PCT search included, under "Documents Considered to Relevant," WO 2018/035059 A1, assigned to Genzyme. This PCT search also included a paper by the inventors of the '377 and '880 patents. *See* Exhibit W (J. Xiaoying et al., *Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins*, HUM. GENE THER. METHODS, June 2017).

61. In the Written Opinion of the International Searching Authority attached to the PCT search, the claims of Sarepta's '595 Application were deemed to lack novelty at least in view of WO 2018/035059 A1. *See* Exhibit V.

62. Genzyme's WO 2018/035059 A1 was PCT/US2017/046814 which was filed in the U.S. as a national phase application under 35 U.S.C. § 371, with U.S. Application No. 16/325,653. *See* Exhibit Q. This application issued as the '377 patent. *See id.* The '880 Patent is a divisional application of U.S. Application No. 16/325,653, filed as application No. PCT/US2017/046814, which issued as the '377 Patent. *See* Exhibit R. The '313 Patent is a continuation of application

No. 18/801,293, which is a divisional application of U.S. application No. 18/321,542, which issued as the '880 Patent. *See* Exhibit S.

63. On information and belief, Sarepta monitors the patent family deriving from WO 2018/035059 A1 and PCT/US2017/046814—which includes the '377, '880, and '313 Patents—at least for purposes of prosecuting the '595 Application.

64. Sarepta has been aware of the '542 Patent since, at the latest, August 22, 2023. *See* Exhibit K.

65. Sarepta has been aware of the '721 Patent since, at the latest, July 26, 2024. *See* Exhibit I.

66. Sarepta has been aware of the '894 Patent since, at the latest, July 26, 2024. *See* Exhibit I.

67. Sarepta has been aware of the '326 Patent since, at the latest, July 26, 2024. *See* Exhibit I.

68. On information and belief, Sarepta has been aware of the application that led to the '377 Patent since April 9, 2021, the date the international search was completed, or in the alternative, at least since June 30, 2023, the date Sarepta filed the international search with the PTO. Sarepta has been aware of the '377 Patent since its issuance on July 11, 2023 and since, at the latest, July 26, 2024. *See* Exhibits I, Q, and V.

69. On information and belief, Sarepta has been aware of the application that led to the '880 Patent since its publication on February 8, 2024, and aware of the '880 Patent since its issuance on October 22, 2024 and at the latest, by October 31, 2024. *See* Exhibits J and R.

70. Sarepta has been aware of the application that led to the '313 Patent since its publication on May 1, 2025, and aware of the '313 Patent since, at the latest, its issuance on May 13, 2025. *See* Exhibits S and X.

COUNT I
INFRINGEMENT OF THE '542 PATENT

71. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 70, above as though fully set forth herein.

72. Plaintiff has all substantial rights in and to the '542 Patent, including the right to assert any claims for past, present, and future infringement of the '542 Patent against Defendants.

73. Defendants have infringed at least one claim of the '542 Patent by making, using, importing, offering for sale, and/or selling Elevidys® in the United States in violation of 35 U.S.C. § 271(a), (b) and/or (c).

74. The '542 Patent has one independent claim, claim 1, which, as of June 15, 2023, has been statutorily disclaimed. Claims 3 and 6 each depend from claim 1, and thus incorporate all the limitations of claim 1. Claim 1 recites:

> A composition for the storage of purified, recombinant adeno-associated virus (AAV) vector particles, comprising:
>
> > purified, recombinant AAV vector particles at a concentration exceeding 1×10^{13} vg/ml up to 6.4×10^{13} vg/ml;
> >
> > a pH buffer, wherein the pH of the composition is between 7.5 and 8.0; and
> >
> > excipients comprising one or more multivalent ions selected from the group consisting of citrate, sulfate, magnesium, and phosphate; wherein the ionic strength of the composition is greater than 200 mM, and wherein the purified AAV vector particles are stored in the composition without significant aggregation.

75. Elevidys® is a pharmaceutical composition for the storage of purified, rAAV vector particles, employing a "serotype rh74 (AAVrh74) based vector containing the ELEVIDYS

micro-dystrophin transgene under the control of the MHCK7 promoter." Exhibit C, § 11 Description. Elevidys® has a "nominal concentration of 1.33 x 10^{13} vg/mL," which is within the claimed range of 1×10^{13} vg/ml up to 6.4×10^{13} vg/ml. Exhibit C, § 11 Description.

76. Elevidys® contains a pH buffer, which is a combination of tromethamine HCl and tromethamine, and wherein the pH of the composition, which can be calculated from its components, is between 7.5 and 8.0. Exhibit C, § 11 Description ("Each vial [of Elevidys®] contains an extractable volume of 10 mL and the following excipients: 200 mM sodium chloride, 13 mM tromethamine HCl, 7 mM tromethamine, 1 mM magnesium chloride, 0.001% poloxamer 188.").

77. The excipients in Elevidys® comprise a multivalent ion selected from the claimed group, magnesium in the form of magnesium chloride, wherein the ionic strength of the composition is greater than 200 mM.

78. On information and belief, Elevidys® is also stored in the composition without significant aggregation. *See* Exhibit G, § 3.2.P.3.2 Batch Formula (Defendants represented to the FDA that the drug product "[v]ials found to have defects, including visible particles are removed."); *id.*, § 3.2.P.5. Control of Drug Product (The Elevidys® drug product release specifications require the analytical testing of certain attributes of the final drug product before it is permitted to enter the market, including the testing of "Particulate Matter." The FDA reviewer commented that this attribute had "[a]cceptable compendial limits."); *id.*, § 3.2.P.2.6 Compatibility ("According to the Applicant, low levels of visible particles were observed in SRP-9001 drug product vials during the 100% visual inspection process in some batches and were rejected."). On information and belief, the FDA would not approve the product if it failed to meet this requirement.

79. Claim 3 depends from claims 1 and 2 and therefore incorporates all of the limitations of claims 1 and 2. Claims 2 and 3 recite:

> 2. The composition of claim 1, further comprising ethylene oxide/propylene oxide block copolymer Pluronic® F68.
>
> 3. The composition of claim 2, wherein the Pluronic® F68 is present at a concentration of 0.001% (w/v).

80. Elevidys® contains ethylene oxide/propylene oxide block copolymer Pluronic® F68, also known as poloxamer, in the amount of 0.001%. *See* Exhibit C, § 11 Description ("Each vial [of Elevidys®] contains . . . 0.001% poloxamer 188.").

81. Claim 6 depends from claim 1 and recites:

> 6. The composition of claim 1, wherein recovery of the purified, recombinant virus particles is at least about 90% following filtration of the composition of said AAV vector particles through a 0.22 μm filter.

82. On information and belief, the recovery of the purified, recombinant virus particles of Elevidys® is at least about 90% following filtration of the Elevidys® composition through a 0.22 μm filter. The manufacturing process for Elevidys® includes sterile filtration. Exhibit G, § 10.A EXECUTIVE SUMMARY, at p. iv. On information and belief, the sterile filtration utilizes a 0.22 μm filter. *See also* Exhibit C, § 2.4 Administration ("Recommended supplies and materials: Syringe infusion pump, 0.2 micron PES* in-line filter, PVC* (non-DEHP*), polyurethan IV infusion tubing and catheter); Exhibit G, § 3.2.P.2.6 Compatibility ("A study to assess the in-use compatibility and effectiveness of a 0.2 μm in-line filter as part of DP administration to remove potential intrinsic particulates in the [drug product], was conducted. . . ."). On information and belief, the yield of Elevidys® following sterile filtration is within plus or minus 10% of the FDA approved drug product specification (i.e., at least 90%).

83. Defendants' manufacture, sale, offer for sale, importation, and/or use of the patented compositions in Elevidys® claimed in the '542 Patent prior to the expiration of the '542 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 3 and 6 of the '542 Patent.

84. Defendants jointly infringe the '542 Patent by contracting with Catalent or other third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with manufacturers including Catalent for the manufacture of Elevidys®. *See, e.g.*, Sarepta Therapeutics, Inc., Quarterly Report (Form 10-Q) p. 22 (May 1, 2024), https://www.sec.gov/Archives/edgar/data/873303/000095017024051234/srpt-20240331.htm ("Sarepta May 2024 10-Q") ("We have adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits by Catalent upon manufacture of Elevidys® and establish the manner and timing of Catalent's performance.

85. Defendants have infringed the '542 Patent by selling and offering to sell Elevidys® to third parties. *See, e.g.*, Exhibit D, June 22, 2023, Accelerated BLA Approval Letter (issuing "U.S. License No. 2308 to Sarepta Therapeutics, Inc." which "authorizes you to introduce or

deliver for introduction into interstate commerce" Elevidys®.); Sarepta 2024 Form 10-K at p. 8 ("We launched ELEVIDYS in the second quarter of 2023."); *id.* at p. 76 (Sarepta recorded more than $200 million in revenue from U.S. sales of Elevidys® in 2023 alone.); Sarepta 2025 Form 10-K at p. 8 ("We launched ELEVIDYS in the second quarter of 2023."); *id.* at p. 74 (Sarepta recorded more than $820 million in revenue from U.S. sales of Elevidys® in 2024 alone.); Sarepta Therapeutics, Inc. Quarterly Report (Form 10-Q), p.13 (May 6, 2025), https://www.sec.gov/ix?doc=/Archives/edgar/data/0000873303/000095017025064412/srpt-20250331.htm at p. 13 (Q1 2025 Elevidys® net revenue of $375 million) ("Sarepta 2025 Form 10-Q").

86. On information and belief, at least as of August 22, 2023, Defendants have actively induced infringement of one or more claims of the '542 Patent, including but not limited to claims 3 and 6, under 35 U.S.C. § 271(b), by providing the infringing product to third parties along with a label providing instructions for use with patients and/or by directing or instructing Catalent or other third parties to manufacture the infringing product, with knowledge of the '542 Patent and that the induced acts would constitute infringement.

87. Moreover, on information and belief, Defendants contribute to infringement of the '542 Patent, including but not limited to claims 3 and 6, under 35 U.S.C. § 271(c) by supplying components of the claimed compositions, such as the provision of engineered rAAV particles for formulation into finished drug product, such components having no substantially non-infringing uses, with knowledge of the '542 Patent and its infringement at least as of August 22, 2023.

88. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '542 Patent.

89. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '542 Patent.

90. Sarepta submitted its BLA for Elevidys® to FDA on September 28, 2022.

91. [redacted]

92. [redacted]

93. [redacted]

94. On June 22, 2023, Sarepta obtained FDA accelerated approval to market Elevidys® for the treatment of ambulatory pediatric patients aged 4 through 5 years with DMD with a confirmed mutation in the DMD gene. *See* Exhibit D, Accelerated BLA Approval.

95. Given Sarepta's knowledge of the '542 Patent, [redacted] and the July and October 2024 letters from Genzyme, Sarepta's continued infringement of the '542 Patent by its manufacture, use, sale, importation, and/or offer to sell Elevidys® is deliberate and intentional.

96. In addition, given Sarepta's knowledge of the '542 Patent, its BLA submissions, and the July and October 2024 letters from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '542 Patent constituted an unjustifiably high risk of infringement of the '542 Patent.

97. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '542 Patent has been and continues to be deliberate, intentional, egregious, willful, and in reckless disregard of the valid patent claims of the '542 Patent and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '542 Patent has been willful.

COUNT II
INFRINGEMENT OF THE '721 PATENT

98. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 97, above as though fully set forth herein.

99. Plaintiff has all substantial rights in and to the '721 Patent, including the right to assert any claims for past, present, and future infringement of the '721 Patent against Defendants.

100. Defendants have infringed at least one claim of the '721 Patent by making, using, importing, offering for sale, and/or selling Elevidys® in the United States in violation of 35 U.S.C. § 271(a), (b) and/or (c).

101. The '721 Patent has one independent claim, claim 1. Claim 1 recites:

> A method of preventing aggregation of recombinant adeno-associated virus (rAAV) virions in a purified preparation of rAAV virions, comprising:
>
> 1) providing a lysate comprising rAAV virions;
>
> 2) purifying rAAV virions from the lysate using ultracentrifugation and/or chromatography, wherein said virions are purified; and
>
> 3) adding one or more salts of multivalent ions selected from the group consisting of citrate, phosphate, sulfate and magnesium to said purified virions to produce a preparation of virions with an ionic strength of at least 200 mM, wherein the concentration of purified rAAV virions in said preparation exceeds 1×10^{13} vg/ml up to 6.4×10^{13} vg/ml; and wherein the pH of the purified preparation of rAAV virions is between 7.5 and 8.0.

102. Elevidys® is a purified preparation of rAAV virions, comprising a "serotype rh74 (AAVrh74) based vector containing the ELEVIDYS micro-dystrophin transgene under the control of the MHCK7 promoter." Exhibit C, § 11. On information and belief, Elevidys® is manufactured using a chromatography-based purification method. Exhibit G, § 3.2.P.2.3 Manufacturing Process Development ("Process B clinical DP is manufactured at Catalent BioPark and has been validated as the intended commercial process. . . ."); *id.,* § 10.A EXECUTIVE SUMMARY ("Process B utilizes a scaled-up purification method that incorporates chromatography-based methods purification of the DP, including separation of the empty capsid residuals from the full capsids.").

103. On information and belief, Elevidys® is manufactured according to a method that prevents aggregation of the rAAV virions within the drug product. *See* Exhibit G, § 3.2.P.3.2

Batch Formula (Defendants represented to the FDA that the drug product "[v]ials found to have defects, including visible particles are removed."); *id.*, § 3.2.P.5. Control of Drug Product (The Elevidys® drug product release specifications require the analytical testing of certain attributes of the final drug product before it is permitted to enter the market, including the testing of "Particulate Matter." The FDA reviewer commented that this attribute had "[a]cceptable compendial limits."); *id.*, § 3.2.P.2.6 Compatibility ("According to the Applicant, low levels of visible particles were observed in SRP-9001 drug product vials during the 100% visual inspection process in some batches and were rejected."). On information and belief, the FDA would not approve Elevidys® if it failed to meet this requirement.

104. On information and belief, Elevidys® is manufactured recombinantly using a cell bank, and the drug substance is purified by providing a lysate containing the rAAV virions. Exhibit G § 3.2.A.2 Adventitious Agents Safety Evaluation ("generation of cell banks and DS manufacturing" were reviewed in "3.2.S.2.3 Control of Materials. The materials are satisfactorily controlled.").

105. On information and belief, the rAAV virions in Elevidys® are purified from the lysate using chromatography. Exhibit G, § 3.2.P.2.3 Manufacturing Process Development ("Process B clinical DP is manufactured at Catalent BioPark and has been validated as the intended commercial process . . . ."); *id.,* § 10.A EXECUTIVE SUMMARY ("Process B utilizes a scaled-up purification method that incorporates chromatography-based methods purification of the DP, including separation of the empty capsid residuals from the full capsids.").

106. Elevidys® is prepared by adding to the purified virions the salt of a multivalent ion, magnesium chloride, selected from the claimed group, to produce a preparation of virions with an ionic strength of at least 200 mM. Exhibit C, § 11 Description ("Each vial [of Elevidys®] contains

an extractable volume of 10 mL and the following excipients: 200 mM sodium chloride, 13 mM tromethamine HCl, 7 mM tromethamine, 1 mM magnesium chloride, 0.001% poloxamer 188.").

107. Elevidys® has a "nominal concentration of 1.33 x 10^{13} vg/mL," which is within the claimed range of 1×10^{13} vg/ml up to 6.4×10^{13} vg/ml. Exhibit C, § 11 Description.

108. The pH of the purified preparation of rAAV virions of Elevidys® is between 7.5 and 8.0. The pH of Elevidys® can be calculated from its components and is between 7.5 and 8.0. Exhibit C, § 11 Description ("Each vial [of Elevidys®] contains an extractable volume of 10 mL and the following excipients: 200 mM sodium chloride, 13 mM tromethamine HCl, 7 mM tromethamine, 1 mM magnesium chloride, 0.001% poloxamer 188.").

109. Claim 7 depends from claim 1 and recites:

> The method of claim 1, wherein, after addition of the one or more salts of multivalent ions, recovery of the virions is at least about 90% following filtration of the preparation of virions through a 0.22 μm filter.

110. On information and belief, the recovery of the purified AAV virions of Elevidys® is at least about 90% recovered following filtration of the Elevidys® composition through a 0.22 μm filter, after the addition of a salt of a multivalent ion, magnesium chloride. The manufacturing process for Elevidys® includes sterile filtration. Exhibit G, § 10.A. EXECUTIVE SUMMARY at p. iv. On information and belief, the sterile filtration utilizes a 0.22 μm filter. *See also* Exhibit C, § 2.4 Administration ("Recommended supplies and materials: Syringe infusion pump, 0.2 micron PES* in-line filter, PVC* (non-DEHP*), polyurethan IV infusion tubing and catheter); Exhibit G, § 3.2.P.2.6 Compatibility ("A study to assess the in-use compatibility and effectiveness of a 0.2 μm in-line filter as part of DP administration to remove potential intrinsic particulates in the [drug product], was conducted."). On information and belief, the yield of Elevidys® following sterile

filtration is within plus or minus 10% of the FDA approved drug product specification (i.e., at least 90%).

111. Defendants' practice of the patented methods claimed in the '721 Patent prior to the expiration of the '721 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 1 and 7 of the '721 Patent.

112. Defendants jointly infringe the '721 Patent by contracting with Catalent or other third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with manufacturers including Catalent for the manufacture of Elevidys®. *See, e.g.*, Sarepta May 2024 10-Q at p. 22 ("We have adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits upon performance by Catalent of the steps of the patented methods of the '721 Patent in the manufacture of Elevidys®, and establish the manner and timing of Catalent's performance.

113. Defendants have infringed the '721 Patent by selling and offering to sell Elevidys®, made by the methods claimed in the '721 Patent to third parties. *See, e.g.*, Exhibit D, June 22, 2023, Accelerated BLA Approval Letter at p. 1 (issuing "U.S. License No. 2308 to Sarepta

Therapeutics, Inc." which "authorizes you to introduce or deliver for introduction into interstate commerce" Elevidys®.); Sarepta 2024 Form 10-K at p. 8 ("We launched ELEVIDYS in the second quarter of 2023."); *id.* at p. 76 (Sarepta recorded more than $200 million in revenue from U.S. sales of Elevidys® in 2023 alone.); Sarepta 2025 Form 10-K at p. 8 ("We launched ELEVIDYS in the second quarter of 2023."); *id.* at p. 74 (Sarepta recorded more than $820 million in revenue from U.S. sales of Elevidys® in 2024 alone.); Sarepta 2025 Form 10-Q at p. 13 (Q1 2025 Elevidys® net revenue of $375 million).

114. On information and belief, at least as of August 22, 2023, Defendants have actively induced Catalent or other third parties to infringe one or more claims of the '721 Patent, including but not limited to claims 1 and 7, under 35 U.S.C. § 271(b), by instructing and contracting with Catalent or other third parties to manufacture Elevidys® in accordance with the claimed methods, with knowledge of the '721 Patent and that the induced acts would constitute infringement.

115. Moreover, on information and belief, Defendants contribute to infringement of the '721 Patent, including but not limited to claims 1 and 7, under 35 U.S.C. § 271(c) by supplying materials or apparatuses for use in practicing the patented method, such as the provision of engineered rAAV virions to manufacture the finished drug product, such materials or apparatuses having no substantially non-infringing uses, with knowledge of the '721 Patent and its infringement at least as of August 22, 2023.

116. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '721 Patent.

117. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '721 Patent.

118. Sarepta submitted its BLA for Elevidys® to FDA on September 28, 2022.

119. [redacted]

120. [redacted]

121. [redacted]

[redacted]

122. On June 22, 2023, Sarepta obtained FDA accelerated approval to market Elevidys® for the treatment of ambulatory pediatric patients aged 4 through 5 years with DMD with a confirmed mutation in the DMD gene. *See* Exhibit D, Accelerated BLA Approval.

123. Given Sarepta's knowledge of the '721 Patent, [redacted] and the July and October 2024 letters from Genzyme, Sarepta's continued infringement of the '721 Patent by its manufacture, use, importation, sale, or offer to sell Elevidys®, or inducing others to manufacture, use, import, sell, or offer to sell Elevidys®, is deliberate and intentional.

124. In addition, given Sarepta's knowledge of the '721 Patent, its BLA submissions, and the July and October 2024 letters from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '721 Patent constituted an unjustifiably high risk of infringement of the '721 Patent.

125. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '721 Patent has been and continues to be deliberate, intentional, egregious, willful, and in reckless disregard of the valid patent claims of the '721 Patent, and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '721 Patent has been willful.

COUNT III
INFRINGEMENT OF THE '894 PATENT

126. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 125 above as though fully set forth herein.

127. Plaintiff has all substantial rights in and to the '894 Patent, including the right to assert any claims for past, present, and future infringement of the '894 Patent against Defendants.

128. Defendants have infringed at least one claim of the '894 Patent by practicing the patented methods during the manufacture of Elevidys® in the United States in violation of 35 U.S.C. §§ 271(a), (b) and/or (c).

129. The '894 Patent has one independent claim, claim 1. Claim 1 recites:

> A method of quantifying one or more species of individual variant viral particles comprising fragmented recombinant adeno-associated viral (rAAV) genomes in a heterogeneous mixture of viral particles, said method comprising:
>
> > (i) subjecting the heterogeneous mixture of viral particles to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate sedimenting boundaries, wherein the boundary sedimentation velocity is from about 3,000 rpm to about 20,000 rpm;
> >
> > (ii) measuring the rate of movement or migration of the sedimenting boundaries, wherein movement or migration of each species of individual viral particles in the heterogeneous mixture of viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable species of viral particle, and wherein the individual viral particles comprise empty particles without genome, particles with full genomes and particles with fragmented genomes;
> >
> > (iii) determining the genome size of one or more species of the individual variant viral particles in the heterogeneous mixture of viral particles; and
> >
> > (iv) determining the quantity of one or more species of the variant viral particles in the heterogeneous mixture of viral particles.

130. On information and belief, Elevidys® is a heterogeneous mixture of viral particles, and Sarepta quantifies one or more species of individual variant viral particles comprising fragmented recombinant adeno-associated viral (rAAV) genomes in Elevidys®. Elevidys® uses a non-replicating, rAAV vector of the serotype rh74 ("rAAVrh74") capsid to package and deliver a human micro-dystrophin transgene under the control of the MHCK7 promoter. *See* Exhibit C, §

11 Description. When rAAV vectors are packaged, a heterogeneous mixture of viral particles is generated.

131. In manufacturing Elevidys® drug product, on information and belief, Defendants calculate the percentage of full capsids in Elevidys® drug product. *See* Exhibit G, CBER CMC BLA Review Memo at 3.2.P.5, Control of Drug Product. As taught in the '894 patent, analytical ultracentrifugation can be used to calculate the percentage of full, empty, and partial capsids in a heterogeneous mixture of viral particles. *See* Exhibit O. As noted by Beckman Coulter, an analytical ultracentrifuge equipment manufacturer, analytical ultracentrifugation is acknowledged as the "gold standard" in the detection and quantification of AAV particles and "a valuable tool to analyze rAAV vectors notwithstanding the composition and length of the transgene or the viral serotype." Exhibit T. Thus, on information and belief, Defendants calculate the percentage of full capsids in Elevidys® drug product, in part, by subjecting it to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate sedimenting boundaries. On information and belief, the boundary sedimentation velocity used by Defendants is from about 3,000 rpm to about 20,000 rpm. *See* Exhibit T (detailing analytical ultracentrifugation analysis on rAAV particles by the inventor run at 20,000 rpm); Exhibit O, Table 1 (showing exemplary rpm ranges of 3,000 to 20,000 rpm).

132. In manufacturing Elevidys® drug product, on information and belief, Defendants measure the rate of movement or migration of the sedimenting boundaries, wherein the movement or migration of each species of individual viral particles in the heterogenous mixture of viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable species of viral particle, and wherein the individual viral particles

comprise empty particles without genome, particles with full genomes and particles with fragmented genomes.

133. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants quantify each resolvable species of viral particle.

134. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants determine the relative percentage of each resolvable species of viral particle.

135. On information and belief, in order to accurately calculate the percentage of full capsids in the product, Defendants determine the genome size of one or more species of the individual variant viral particles.

136. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants must determine the quantity of one or more species of the variant viral particles in the heterogeneous mixture of viral particles.

137. Claim 20 depends from claim 1 therefore incorporates all of the limitations of claim 1. Claim 20 recites:

> 20. The method of claim 1, wherein the total concentration of viral particles in the heterogeneous mixture of viral particles prior to step (i) is greater than 5×10^{11} vg/mL.

138. Elevidys® has a "nominal concentration of 1.33 x 10^{13} vg/mL" following step (i) of claim 1, so the concentration prior to step (i) is within the claimed range of greater than 5×10^{11} vg/ml. Exhibit C, § 11 Description.

139. On information and belief, Defendants perform each and every step of at least the methods claimed in claims 1 and 20 of the '894 Patent at least during release testing prior to

releasing any batch of Elevidys® for commercial sale. On information and belief, this testing is required for commercialization of Elevidys®.

140. Defendants' use of the patented methods during the manufacture of Elevidys® as claimed in the '894 Patent prior to the expiration of the '894 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 1 and 20 of the '894 Patent.

141. Defendants jointly infringe the '894 Patent by contracting with third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with third-party manufacturers and testing companies for the manufacture, testing, and sale of Elevidys®. *See, e.g.*, Sarepta May 2024 10-Q at p. 22 ("We have adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits by third parties upon manufacture of Elevidys®, and establish the manner and timing of those third parties' performance.

142. On information and belief, at least as of July 26, 2024, Defendants have actively induced third parties to infringe one or more claims of the '894 Patent, including but not limited

to claims 1 and 20, under 35 U.S.C. § 271(b), by instructing and contracting with third parties to manufacture Elevidys® in accordance with the claimed methods, with knowledge of the '894 Patent and that the induced acts would constitute infringement.

143. Moreover, on information and belief, Defendants contribute to infringement of the '894 Patent, including but not limited to claims 1 and 20, under 35 U.S.C. § 271(c) by supplying materials or apparatuses for use in practicing the patented method, such as the provision of engineered rAAV virions to manufacture the finished drug product, such materials or apparatuses having no substantially non-infringing uses, with knowledge of the '894 Patent and its infringement at least as of July 26, 2024.

144. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '894 Patent.

145. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '894 Patent.

146. Given Sarepta's knowledge of the '894 Patent from the July and October 2024, and May 2025, letters from Genzyme, Sarepta's continued infringement of the '894 Patent by its manufacture, use, importation, sale, or offer to sell Elevidys®, or inducing others to manufacture, use, import, sell, or offer to sell Elevidys®, is deliberate and intentional.

147. In addition, given Sarepta's knowledge of the '894 Patent from the July and October 2024, and May 2025, letters from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '894 Patent constituted an unjustifiably high risk of infringement of the '894 Patent.

148. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '894 Patent has been and continues to be deliberate, intentional, egregious,

willful, and in reckless disregard of the valid patent claims of the '894 Patent, and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '894 Patent has been willful.

COUNT IV
INFRINGEMENT OF THE '326 PATENT

149. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 148 above as though fully set forth herein.

150. Plaintiff has all substantial rights in and to the '326 Patent, including the right to assert any claims for past, present, and future infringement of the '326 Patent against Defendants.

151. Defendants have infringed at least one claim of the '326 Patent by using the patented methods during the manufacture of Elevidys® in the United States in violation of 35 U.S.C. §§ 271(a), (b) and/or (c).

152. The '326 Patent has two independent claims, including claim 1. Claim 1 recites:

> A method of determining the size of one or more fragmented genomes in a preparation of viral particles comprising recombinant adeno-associated viral (rAAV) vectors encapsidated into viral capsids, said method comprising:
>
> > (i) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate one or more sedimenting boundaries, wherein the boundary sedimentation velocity is from about 3,000 rpm to about 20,000 rpm;
> >
> > (ii) measuring the rate of movement or migration of the one or more sedimenting boundaries, wherein movement or migration of the viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable viral particle, and wherein one or more of the viral particles comprise a fragmented genome, and determining the sedimentation coefficients of the viral particles comprising one or more of the fragmented genomes in the preparation; and
> >
> > (iii) determining the size of the one or more fragmented genomes as a function of the sedimentation coefficients of the viral particles comprising the one or more fragmented genomes.

153. On information and belief, Elevidys® is a preparation of viral particles comprising rAAV genomes encapsidated into viral vectors, and Sarepta determines the size of one or more fragmented genomes in Elevidys®. Elevidys® uses a non-replicating, rAAV vector of the serotype rh74 ("rAAVrh74") capsid to package and deliver a human micro-dystrophin transgene under the control of the MHCK7 promoter. *See* Exhibit C, § 11 Description.

154. In manufacturing Elevidys® drug product, on information and belief, Defendants calculate the percentage of full capsids in Elevidys® drug product. *See* Exhibit G, CBER CMC BLA Review Memo at 3.2.P.5, Control of Drug Product. As taught in the '326 patent, analytical ultracentrifugation can be used to calculate the percentage of full, empty, and partial capsids in a heterogeneous mixture of viral particles. *See* Exhibit P. As noted by Beckman Coulter, an analytical ultracentrifuge equipment manufacturer, analytical ultracentrifugation is acknowledged as the "gold standard" in the detection and quantification of AAV particles and "a valuable tool to analyze rAAV vectors notwithstanding the composition and length of the transgene or the viral serotype." Exhibit T. Thus, on information and belief, Defendants calculate the percentage of full capsids in Elevidys® drug product, in part, by subjecting it to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate sedimenting boundaries. On information and belief, the boundary sedimentation velocity used by Defendants is from about 3,000 rpm to about 20,000 rpm. *See* Exhibit T (detailing analytical ultracentrifugation analysis on rAAV particles by the inventor run at 20,000 rpm); Exhibit P, Table 1 (showing exemplary rpm ranges of 3,000 to 20,000 rpm).

155. In manufacturing Elevidys® drug product, on information and belief, Defendants measure the rate of movement or migration of the one or more sedimenting boundaries, wherein movement or migration of the viral particles results in distinct sedimenting boundaries, each

distinct sedimenting boundary corresponding to a resolvable viral particle, and wherein one or more of the viral particles comprise a fragmented genome, and determine the sedimentation coefficients of the viral particles comprising one or more of the fragmented genomes in the preparation.

156. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants quantify each resolvable species of viral particle.

157. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants determine the size of the one or more fragmented genomes as a function of the sedimentation coefficients of the viral particles comprising the one or more fragmented genomes.

158. Claim 12 depends from claim 1 therefore incorporates all of the limitations of claim 1. Claim 12 recites:

> 12. The method of claim 1, wherein the total concentration of viral particles in the AAV vector preparation prior to step (i) is greater than 5×10^{11} vg/mL.

159. Elevidys® has a "nominal concentration of 1.33 x 10^{13} vg/mL" following step (i) of claim 1, so the concentration prior to step (i) is within the claimed range greater than 5×10^{11} vg/ml. Exhibit C, § 11 Description.

160. Independent claim 20 recites:

> A method of determining the molar concentrations of each species of individual viral particles in a heterogeneous mixture of viral particles comprising recombinant adeno-associated viral (rAAV) vectors encapsidated into viral capsids, said method comprising:
>
> (i) subjecting the heterogeneous mixture of viral particles to analytical ultracentrifugation under boundary sedimentation velocity conditions to

> generate sedimenting boundaries, wherein the boundary sedimentation velocity is from about 3,000 rpm to about 20,000 rpm;
>
> (ii) measuring the rate of movement or migration of the sedimenting boundaries, wherein movement or migration of each species of the individual viral particles in the heterogeneous mixture of viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable species of viral particle, and wherein the heterogeneous mixture of viral particles comprises full genomes, fragmented genomes and empty capsids without genome;
>
> (iii) determining the sedimentation coefficients of each species of the individual viral particles in the heterogeneous mixture of viral particles; and
>
> (iv) quantifying the molar concentration of each species of the individual viral particles in the heterogeneous mixture of viral particles.

161. On information and belief, Elevidys® is a preparation of a heterogeneous mixture of viral particles comprising rAAV vectors encapsidated into viral capsids and Sarepta determines the molar concentrations of each species of individual viral particles in the mixture. Elevidys® uses a non-replicating, rAAV vector of the serotype rh74 ("rAAVrh74") capsid to package and deliver a human micro-dystrophin transgene under the control of the MHCK7 promoter. *See* Exhibit C, § 11 Description. When rAAV vectors are packaged, a heterogeneous mixture of viral particles is generated.

162. In manufacturing Elevidys® drug product, on information and belief, Defendants calculate the percentage of full capsids in Elevidys® drug product. *See* Exhibit G, CBER CMC BLA Review Memo at 3.2.P.5, Control of Drug Product. As taught in the '894 patent, analytical ultracentrifugation can be used to calculate the percentage of full, empty, and partial capsids in a heterogeneous mixture of viral particles. *See* Exhibit P. As noted by Beckman Coulter, an analytical ultracentrifuge equipment manufacturer, analytical ultracentrifugation is acknowledged as the "gold standard" in the detection and quantification of AAV particles and "a valuable tool to analyze rAAV vectors notwithstanding the composition and length of the transgene or the viral

serotype." Exhibit T. Thus, on information and belief, Defendants calculate the percentage of full capsids in Elevidys® drug product, in part, by subjecting it to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate sedimenting boundaries. On information and belief, the boundary sedimentation velocity used by Defendants is from about 3,000 rpm to about 20,000 rpm. *See* Exhibit T (detailing analytical ultracentrifugation analysis on rAAV particles by the inventor run at 20,000 rpm); Exhibit P, Table 1 (showing exemplary rpm ranges of 3,000 to 20,000 rpm).

163. In manufacturing Elevidys® drug product, on information and belief, Defendants measure the rate of movement or migration of the sedimenting boundaries, wherein movement or migration of each species of the individual viral particles in the heterogeneous mixture of viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable species of viral particle, and wherein the heterogeneous mixture of viral particles comprises full genomes, fragmented genomes and empty capsids without genome.

164. In manufacturing Elevidys® drug product, on information and belief, Defendants determine the sedimentation coefficients of each species of the individual viral particles in the heterogeneous mixture of viral particles.

165. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants quantify each resolvable species of viral particle.

166. On information and belief, in order to accurately calculate the percentage of full capsids using analytical ultracentrifugation, Defendants quantify the molar concentration of each species of the individual viral particles in the heterogeneous mixture of viral particles.

167. On information and belief, Defendants perform each and every step of at least the methods claimed in claims 1, 12 and 20 of the '326 Patent at least during release testing prior to releasing any batch of Elevidys® for commercial sale. On information and belief, release testing is required for commercialization of Elevidys®.

168. Defendants' use of the patented methods during the manufacture of Elevidys® as claimed in the '326 Patent prior to the expiration of the '326 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 1, 12, and 20 of the '326 Patent.

169. Defendants jointly infringe the '326 Patent by contracting with third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with third-party manufacturers and testing companies for the manufacture, testing, and sale of Elevidys®. *See, e.g.*, Sarepta May 2024 10-Q at p. 22 ("We have adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits by third parties upon manufacture of Elevidys®, and establish the manner and timing of those third parties' performance.

170. On information and belief, at least as of July 26, 2024, Defendants have actively induced third parties to infringe one or more claims of the '326 Patent, including but not limited to claims 1, 12, and 20, under 35 U.S.C. § 271(b), by instructing and contracting with third parties to manufacture Elevidys® in accordance with the claimed methods, with knowledge of the '326 Patent and that the induced acts would constitute infringement.

171. Moreover, on information and belief, Defendants contribute to infringement of the '326 Patent, including but not limited to claims 1, 12, and 20 under 35 U.S.C. § 271(c) by supplying materials or apparatuses for use in practicing the patented method, such as the provision of engineered rAAV virions to manufacture the finished drug product, such materials or apparatuses having no substantially non-infringing uses, with knowledge of the '326 Patent and its infringement at least as of July 26, 2024.

172. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '326 Patent.

173. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '326 Patent.

174. Given Sarepta's knowledge of the '326 Patent from the July and October 2024, and May 2025, letters from Genzyme, Sarepta's continued infringement of the '326 Patent by its manufacture, use, importation, sale, or offer to sell Elevidys®, or inducing others to manufacture, use, import, sell, or offer to sell Elevidys®, is deliberate and intentional.

175. In addition, given Sarepta's knowledge of the '326 Patent from the July and October 2024, and May 2025, letters from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '326 Patent constituted an unjustifiably high risk of infringement of the '326 Patent.

176. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '326 Patent has been and continues to be deliberate, intentional, egregious, willful, and in reckless disregard of the valid patent claims of the '326 Patent, and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '326 Patent has been willful.

COUNT V
INFRINGEMENT OF THE '377 PATENT

177. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 176 above as though fully set forth herein.

178. Plaintiff has all substantial rights in and to the '377 Patent, including the right to assert any claims for past, present, and future infringement of the '377 Patent against Defendants.

179. Defendants have infringed at least one claim of the '377 Patent by practicing the patented methods during the manufacture of Elevidys® in the United States in violation of 35 U.S.C. §§ 271(a), (b) and/or (c).

180. The '377 Patent has two independent claims. Claim 1 recites:

> A method to determine the serotype of an adeno-associated virus (AAV) particle comprising:
>
> (a) denaturing the AAV particle,
>
> (b) directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and
>
> (c) determining the masses of VP1, VP2, and VP3 of the AAV particle;
>
> wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype,
>
> and wherein the method is performed in the absence of a gel separation step.

181. Defendants are required to report the identity of the AAV vector capsid and the capsid purity in Elevidys® as part of its Drug Product release specification. *See* Exhibit G § 3.2.P.5, Table 100.

182. On information and belief, the methods disclosed by Sarepta in its '595 Application are used "to determine the serotype of an AAV particle at least based in part on the ratio of VP1, VP2 and VP3 capsid proteins in an AAV particle and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins, wherein the ratio of VP1, VP2 and VP3 capsid proteins and the masses of one or more of the VP1, VP2 and VP3 capsid proteins are determined by the methods disclosed herein." *See* Exhibit U at [0097].

183. On information and belief, Defendants determine the serotype of Elevidys® at least during release testing prior to commercial sale. On information and belief, serotype testing is required for commercialization of Elevidys®.

184. Sarepta's method denatures the AAV particles. *See* Exhibit U at [0010] ("In some aspects, the capsids on the AAV particle is denatured into the individual VP1, VP2 and VP3 proteins in the column of the liquid chromatography. In some aspects, the capsid proteins are separated by the liquid chromatography."); [0174] ("Here, an AAV particle was denatured and separated to the VP1, VP2 and VP3 capsid proteins in liquid chromatography.").

185. Sarepta's method directly subjects the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis. *See* Exhibit U at [0098] ("Mass Spectrometry is an analytical technique for protein characterization. In some aspects, a method for the characterization of the AAVrh74 capsid protein ratio along with the intact mass for all three capsid proteins by liquid chromatography and mass spectrometry is provided."); [0214] ("Table 10 three lots of Host Cell Protein analyzed by LC/MS").

186. Sarepta's method determines the masses of VP1, VP2, and VP3 of the AAV particle. *See* Exhibit U at [0008] ("The methods disclosed herein are used to determine the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins."); [0186] ("The VP1, VP2 and VP3 capsid proteins separated in the liquid chromatography were first subjected to UV to determine the relative amounts and then to mass spectrometry to determine the masses of the VP1, VP2 and VP3 capsid proteins.")

187. On information and belief, Defendants use the methods described in the '595 Application, and particularly Example 4, to analyze the specific combination of masses of VP1, VP2 and VP3 for purposes of determining the AAV serotype of Elevidys® during release testing.

188. On information and belief, Sarepta performs the methods described in the '595 Application, and particularly Example 4, without gel separation. There is no reference to gel separation in the specification or claims of the '595 Application, and the methods recited in the '595 Application are directed to intact protein analysis. On information and belief, Sarepta's LC-MS intact mass method for Elevidys® is performed without gel separation.

189. Claim 4 of the '377 Patent depends from claim 1, and further recites "wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent." Sarepta's method denatures using guanidine hydrochloride. *See* Exhibit U at [0017] ("In some aspects, the protein can be denatured using reagents like Guanidine and Urea."); [0196] ("The samples were first denatured by performing a buffer exchange into 6M Guanidine Hydrochloride, 20 mM Tris-HCl, pH 7.5.").

190. Claim 6 of the '377 Patent depends from claim 1, and further recites "wherein the liquid chromatography is reverse phase chromatography." Sarepta's method uses reverse phase chromatography. *See* Exhibit U at [0024] ("In some aspects, the liquid chromatography is a

reverse phase liquid chromatography."); cl. 4 ("The method of claim 1, wherein the liquid chromatography is a reverse phase liquid chromatography.").

191. Claim 7 of the '377 Patent depends from claim 6, and further recites "wherein the reverse phase chromatography is a C4 or C8 reverse chromatography." Sarepta's method uses C4 or C8 reverse chromatography. *See* Exhibit U at [0024] ("In some aspects, the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column."); cl. 5 ("The method of claim 4, wherein the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column.").

192. On information and belief, Defendants perform each and every step of at least the methods claimed in claims 1, 4, 6, and 7 of the '377 Patent at least during release testing prior to releasing any batch of Elevidys® for commercial sale. On information and belief, this testing is required for commercialization of Elevidys®. To the extent Defendants do not perform the methods as specifically set forth in the '595 Application, on information and belief, Sarepta performs a substantially similar method that includes each and every step of at least the methods claimed in claims 1, 4, 6, and 7 of the '377 Patent.

193. Defendants' use of the patented methods during the manufacture of Elevidys® as claimed in the '377 Patent prior to the expiration of the '377 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 1, 4, 6, and 7 of the '377 Patent.

194. Defendants jointly infringe the '377 Patent by contracting with third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with third-party manufacturers and testing companies for the manufacture, testing, and sale of Elevidys®. *See, e.g.*, Sarepta May 2024 10-Q at p. 22 ("We have

adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits by third parties upon manufacture of Elevidys®, and establish the manner and timing of those third parties' performance.

195. On information and belief, at least as of July 11, 2023, Defendants have actively induced third parties to infringe one or more claims of the '377 Patent, including but not limited to claims 1, 2, 4 and 7, under 35 U.S.C. § 271(b), by instructing and contracting with third parties to manufacture Elevidys® in accordance with the claimed methods, with knowledge of the '377 Patent and that the induced acts would constitute infringement.

196. Moreover, on information and belief, Defendants contribute to infringement of the '377 Patent, including but not limited to claims 1, 2, 4 and 7, under 35 U.S.C. § 271(c) by supplying materials or apparatuses for use in practicing the patented method, such as the provision of engineered rAAV virions to manufacture the finished drug product, such materials or apparatuses having no substantially non-infringing uses, with knowledge of the '377 Patent and its infringement at least as of July 11, 2023.

197. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '377 Patent.

198. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '377 Patent.

199. Given Sarepta's knowledge of the '377 Patent from the July and October 2024, and May 2025, letters from Genzyme, Sarepta's continued infringement of the '377 Patent by its manufacture, use, importation, sale, or offer to sell Elevidys®, or inducing others to manufacture, use, import, sell, or offer to sell Elevidys®, is deliberate and intentional.

200. In addition, given Sarepta's knowledge of the '377 Patent from the July and October 2024, and May 2025, letters from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '377 Patent constituted an unjustifiably high risk of infringement of the '377 Patent.

201. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '377 Patent has been and continues to be deliberate, intentional, egregious, willful, and in reckless disregard of the valid patent claims of the '377 Patent, and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '377 Patent has been willful.

COUNT VI
INFRINGEMENT OF THE '880 PATENT

202. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 201 above as though fully set forth herein.

203. Plaintiff has all substantial rights in and to the '880 Patent, including the right to assert any claims for past, present, and future infringement of the '880 Patent against Defendants.

204. Defendants have infringed at least one claim of the '880 Patent by using the patented methods during the manufacture of Elevidys® in the United States in violation of 35 U.S.C. §§ 271(a), (b) and/or (c).

205. The '880 Patent has three independent claims. Claim 1 recites:

> A method of analyzing a preparation of AAV particles, the method comprising:
>
> (a) denaturing the AAV particles,
>
> (b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and
>
> (c) determining the masses of one or more viral proteins (VPs) of the particles in the preparation;
>
> wherein the method is performed in the absence of a gel separation step.

206. Defendants are required to report analysis of the capsid purity, potency, and vector genome concentration in Elevidys® as part of its Drug product release specification. *See* Exhibit G § 3.2.P.5, Table 100.

207. Elevidys® is a preparation of rAAV particles. On information and belief, the methods disclosed by Sarepta in its '595 Application are used to analyze Elevidys®. *See* Exhibit U at Title ("Methods for Analyzing AAV Capsid Proteins"); cl. 1 ("A method to characterize VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle . . . .").

208. Sarepta's method denatures the AAV particles. *See* Exhibit U at [0010] ("In some aspects, the capsids on the AAV particle is denatured into the individual VP1, VP2 and VP3 proteins in the column of the liquid chromatography. In some aspects, the capsid proteins are separated by the liquid chromatography."); [0174] ("Here, an AAV particle was denatured and separated to the VP1, VP2 and VP3 capsid proteins in liquid chromatography.").

209. Sarepta's method subjects the denatured AAV particles to LC/MS intact protein analysis. *See* Exhibit U at [0098] ("Mass Spectrometry is an analytical technique for protein characterization. In some aspects, a method for the characterization of the AAVrh74 capsid protein ratio along with the intact mass for all three capsid proteins by liquid chromatography and mass spectrometry is provided."); *id.* at Example 4, [0189] (LC/MS working example with "detection of post-translation modification of VP1, VP2, and VP3" and "intact mass analysis of AAV.rh74 capsid proteins.").

210. Sarepta's method determines the masses of one or more viral proteins (VPs) of the particles in the preparation of Elevidys®. *See* Exhibit U at [0008] ("The methods disclosed herein are used to determine the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins."); [0186] ("The VP1, VP2 and VP3 capsid proteins separated in the liquid chromatography were first subjected to UV to determine the relative amounts and then to mass spectrometry to determine the masses of the VP1, VP2 and VP3 capsid proteins.")

211. On information and belief, Sarepta performs the methods described in the '595 Application, and particularly Example 4, without gel separation. There is no reference to gel separation in the specification or claims of the '595 Application, and the methods recited in the '595 Application are directed to intact protein analysis. On information and belief, Sarepta's LC-MS intact mass method for Elevidys® is performed without gel separation.

212. Claim 3 of the '880 Patent depends from claim 1, and further recites "wherein the AAV particles are denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent." Sarepta's method denatures with guanidine hydrochloride. *See* Exhibit U at [0017] ("In some aspects, the protein can be denatured using reagents like Guanidine and Urea."); [0196] ("The

samples were first denatured by performing a buffer exchange into 6M Guanidine Hydrochloride, 20 mM Tris-HCl, pH 7.5.").

213. Claim 5 of the '880 Patent depends from claim 1, and further recites "wherein the liquid chromatography is reverse phase liquid chromatography." Sarepta's method uses reverse phase liquid chromatography. *See* Exhibit U at [0024] ("In some aspects, the liquid chromatography is a reverse phase liquid chromatography."); cl. 4 ("The method of claim 1, wherein the liquid chromatography is a reverse phase liquid chromatography.").

214. Claim 6 of the '880 Patent depends from claim 5, and further recites "wherein the reverse phase chromatography is performed with a C4 column." Sarepta's method uses a C4 column. *See* Exhibit U at [0024] ("In some aspects, the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column."); cl. 5 ("The method of claim 4, wherein the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column.").

215. Claim 10 of the '880 Patent recites:

> A method of determining post-translational modifications of viral proteins (VPs) in a preparation of viral particles, the method comprising
>
> a) denaturing the viral particles,
>
> b) subjecting the denatured viral particles to liquid chromatography/mass spectroscopy (LC/MS) intact protein analysis, and
>
> c) determining the masses of one or more VPs of the viral particles
>
> wherein a deviation of one or more of the masses of the one or more VPs from the theoretical masses of VPs that have not undergone post-translational modifications is indicative of post-translational modifications of the VPs,
>
> and wherein the method is performed in the absence of a gel separation step.

216. Elevidys® is a preparation of rAAV particles. On information and belief, the methods disclosed by Sarepta in its '595 Application are used to determine post-translational modifications of viral proteins (VPs) in Elevidys®. *See* Exhibit U at [0018] ("In some aspects, the method further includes determining post translational modification of at least one of VP1, VP2 and VP3 capsid proteins. In some aspects, the method further includes post translational phosphorylation or acetylation of at least one of VP1, VP2 and VP3 capsid proteins."); [0189] ("FIG. 3 shows detection of post-translation modification of VP1, VP2, and VP3.").

217. Sarepta's method denatures the viral particles. *See* Exhibit U at [0010] ("In some aspects, the capsids on the AAV particle is denatured into the individual VP1, VP2 and VP3 proteins in the column of the liquid chromatography. In some aspects, the capsid proteins are separated by the liquid chromatography."); [0174] ("Here, an AAV particle was denatured and separated to the VP1, VP2 and VP3 capsid proteins in liquid chromatography.").

218. Sarepta's method subjects the denatured viral particles to LC/MS intact protein analysis. *See* Exhibit U at [0098] ("Mass Spectrometry is an analytical technique for protein characterization. In some aspects, a method for the characterization of the AAVrh74 capsid protein ratio along with the intact mass for all three capsid proteins by liquid chromatography and mass spectrometry is provided."); *id.* at Example 4, [0189] (LC/MS working example with "detection of post-translation modification of VP1, VP2, and VP3" and "intact mass analysis of AAV.rh74 capsid proteins.").

219. Sarepta's method determines the masses of one or more VPs of the viral particles in the preparation of Elevidys®. *See* Exhibit U at [0008] ("The methods disclosed herein are used to determine the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins."); [0186] ("The VP1, VP2 and VP3 capsid

proteins separated in the liquid chromatography were first subjected to UV to determine the relative amounts and then to mass spectrometry to determine the masses of the VP1, VP2 and VP3 capsid proteins.")

220. On information and belief, Sarepta performs the methods described in the '595 Application, and particularly Example 4, without gel separation. There is no reference to gel separation in the specification or claims of the '595 Application, and the methods recited in the '595 Application are directed to intact protein analysis. On information and belief, Sarepta's LC-MS intact mass method for Elevidys® is performed without gel separation.

221. Claim 13 of the '880 Patent depends from claim 10, and further recites "wherein the liquid chromatography is reverse phase chromatography." Sarepta's method uses reverse phase chromatography. *See* Exhibit U at [0024] ("In some aspects, the liquid chromatography is a reverse phase liquid chromatography."); cl. 4 ("The method of claim 1, wherein the liquid chromatography is a reverse phase liquid chromatography.").

222. Claim 14 of the '880 Patent depends from claim 5, and further recites "wherein the reverse phase chromatography is a C4 or C8 reverse chromatography." Sarepta's method uses C4 or C8 reverse chromatography. *See* Exhibit U at [0024] ("In some aspects, the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column."); cl. 5 ("The method of claim 4, wherein the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column.").

223. Defendants' use of the patented methods during the manufacture of Elevidys® as claimed in the '880 Patent prior to the expiration of the '880 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 1, 3, 5, 6, 10, 13, and 14 of the '880 Patent.

224. On information and belief, Sarepta performs each and every step of at least the methods claimed 1, 3, 5, 6, 10, 13, and 14 of the '880 Patent at least during release testing prior to releasing any batch of Elevidys® for commercial sale. On information and belief, this testing is required for commercialization of Elevidys®. To the extent Defendants do not perform the methods as specifically set forth in the '595 Application, on information and belief, Sarepta performs a substantially similar method that includes each and every step of at least the methods claimed in claims 1, 3, 5, 6, 10, 13, and 14 of the '880 Patent.

225. Defendants jointly infringe the '880 Patent by contracting with third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with manufacturers and testing companies for the manufacture, testing, and sale of Elevidys®. *See, e.g.*, Sarepta May 2024 10-Q at p. 22 ("We have adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits by third parties upon manufacture of Elevidys®, and establish the manner and timing of those third parties' performance.

226. On information and belief, at least as of October 22, 2024, Defendants have actively induced third parties to infringe one or more claims of the '880 Patent, including but not limited to claims 1, 3, 5, 6, 10, 13, and 14 under 35 U.S.C. § 271(b), by instructing and contracting with third parties to manufacture Elevidys® in accordance with the claimed methods, with knowledge of the '880 Patent and that the induced acts would constitute infringement.

227. Moreover, on information and belief, Defendants contribute to infringement of the '880 Patent, including but not limited to claims 1, 3, 5, 6, 10, 13, and 14 under 35 U.S.C. § 271(c) by supplying materials or apparatuses for use in practicing the patented method, such as the provision of engineered rAAV virions to manufacture the finished drug product, such materials or apparatuses having no substantially non-infringing uses, with knowledge of the '880 Patent and its infringement at least as of October 22, 2024. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '880 Patent.

228. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '880 Patent.

229. Given Sarepta's knowledge of the '880 Patent from the October 2024 and May 2025 letters from Genzyme, Sarepta's continued infringement of the '880 Patent by its manufacture, use, importation, sale, or offer to sell Elevidys®, or inducing others to manufacture, use, import, sell, or offer to sell Elevidys®, is deliberate and intentional.

230. In addition, given Sarepta's knowledge of the '880 Patent from the October 2024 and May 2025 letters from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '880 Patent constituted an unjustifiably high risk of infringement of the '880 Patent.

231. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '880 Patent has been and continues to be deliberate, intentional, egregious, willful, and in reckless disregard of the valid patent claims of the '880 Patent, and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '880 Patent has been willful.

COUNT VII
INFRINGEMENT OF THE '313 PATENT

232. Plaintiff repeats and realleges the allegations set forth in paragraphs 1 through 231 above as though fully set forth herein.

233. Plaintiff has all substantial rights in and to the '313 Patent, including the right to assert any claims for past, present, and future infringement of the '313 Patent against Defendants.

234. Defendants have infringed at least one claim of the '313 Patent by using the patented methods during the manufacture of Elevidys® in the United States in violation of 35 U.S.C. §§ 271(a), (b) and/or (c).

235. The '313 Patent has three independent claims. Claim 20 recites:

> A method of preparing a pharmaceutical composition of adeno-associated virus (AAV) particles, the method comprising:
>
> monitoring AAV particles for consistency and/or identity;
>
> wherein the AAV particles comprise viral proteins (VPs) comprising VP1, VP2 and VP3 capsid proteins of an AAV particle capsid,
>
> wherein the AAV particle is monitored for consistency and/or identity by:
>
> a) extracting an aliquot of an AAV particle preparation;
>
> b) denaturing the AAV particles;
>
> c) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;

d) determining the masses of one or more VPs of the AAV particles; and

e) comparing the determined masses of the one or more VPs to theoretical masses of corresponding VPs, wherein the theoretical masses of corresponding VPs are those VPs of known AAV serotypes and/or those that have not undergone undesired post-translational modifications; and

f) determining if there is any deviation of the determined masses of the one or more VPs from the theoretical masses of the corresponding VPs;

wherein the determination of any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs thereby monitors the AAV particles for consistency and/or identity;

wherein the monitoring for consistency and/or identity is performed in the absence of a gel separation step; and

wherein if less than an undesirable amount of deviation is determined during the monitoring for consistency and/or identity, the AAV particles are combined with one or more pharmaceutically acceptable excipients to form the pharmaceutical composition.

236. Elevidys® is a pharmaceutical composition of rAAV vector particles. Exhibit C, § 11 Description.

237. Defendants are required to monitor the AAV particles in Elevidys® for consistency and/or identity and must report analysis of the capsid purity, potency, and vector genome concentration in Elevidys® as part of its Drug product release specification. *See* Exhibit G § 3.2.P.5, Table 100.

238. On information and belief, the methods disclosed by Sarepta in its '595 Application are used to analyze Elevidys® for quality, consistency, and/or identity. *See* Exhibit U at Title ("Methods for Analyzing AAV Capsid Proteins"); cl. 1 ("A method to characterize VP1, VP2, and VP3 capsid proteins in an adeno-associated virus (AAV) particle . . ."); [0007] ("[T]he accurate

measurement of the ratio among the three capsid proteins is important in the AAV vector quality control."); [0214] ("The identity and relative quantity of each residual protein were calculated against the amount of the amount of spike protein standards.")

239. On information and belief, the methods disclosed by Sarepta in its '595 Application are used to analyze Elevidys® viral proteins (VPs) comprising VP1, VP2 and VP3 capsid proteins of an AAV particle capsid. *See* Exhibit U at [0008] ("The methods disclosed herein are used to determine the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins."); [0186] ("The VP1, VP2 and VP3 capsid proteins separated in the liquid chromatography were first subjected to UV to determine the relative amounts and then to mass spectrometry to determine the masses of the VP1, VP2 and VP3 capsid proteins.")

240. Sarepta's method denatures the AAV particles. *See* Exhibit U at [0010] ("In some aspects, the capsids on the AAV particle is denatured into the individual VP1, VP2 and VP3 proteins in the column of the liquid chromatography. In some aspects, the capsid proteins are separated by the liquid chromatography."); [0174] ("Here, an AAV particle was denatured and separated to the VP1, VP2 and VP3 capsid proteins in liquid chromatography.").

241. Sarepta's method subjects the denatured AAV particles to LC/MS intact protein analysis. *See* Exhibit U at [0098] ("Mass Spectrometry is an analytical technique for protein characterization. In some aspects, a method for the characterization of the AAVrh74 capsid protein ratio along with the intact mass for all three capsid proteins by liquid chromatography and mass spectrometry is provided."); *id.* at Example 4, [0189] (LC/MS working example with "detection of post-translation modification of VP1, VP2, and VP3" and "intact mass analysis of AAV.rh74 capsid proteins.").

242. Sarepta's method determines the masses of one or more viral proteins (VPs) of the particles in the preparation of Elevidys®. *See* Exhibit U at [0008] ("The methods disclosed herein are used to determine the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins."); [0186] ("The VP1, VP2 and VP3 capsid proteins separated in the liquid chromatography were first subjected to UV to determine the relative amounts and then to mass spectrometry to determine the masses of the VP1, VP2 and VP3 capsid proteins.")

243. Sarepta's method compares the determined masses of the one or more VPs to theoretical masses of corresponding VPs, wherein the theoretical masses of corresponding VPs are those VPs of known AAV serotypes and/or those that have not undergone undesired post-translational modifications, followed by determining if there is any deviation of the determined masses of the one or more VPs from the theoretical masses of the corresponding VPs. *See* Exhibit U at Table 6 (comparing determined masses to theoretical masses for VP1, VP2, and VP3 for detection of post-translational modifications); [0097] (comparison of VP1, VP2, and VP3 masses for serotype determination).

244. On information and belief, Sarepta's determination of any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs is used to monitor the Elevidys® AAV particles for consistency and/or identity. *See* Exhibit F; Exhibit G.

245. On information and belief, Sarepta performs the methods described in the '595 Application, and particularly Example 4, without gel separation. There is no reference to gel separation in the specification or claims of the '595 Application, and the methods recited in the

'595 Application are directed to intact protein analysis. On information and belief, Sarepta's LC-MS intact mass method for Elevidys® is performed without gel separation.

246. On information and belief, if less than an undesirable amount of deviation is determined during the monitoring of Elevidys® for consistency and/or identity, the AAV particles are combined with one or more pharmaceutically acceptable excipients to form the Elevidys® pharmaceutical composition. *See* Exhibit C, § 11 Description ("Each vial [of Elevidys®] contains an extractable volume of 10 mL and the following excipients: 200 mM sodium chloride, 13 mM tromethamine HCl, 7 mM tromethamine, 1 mM magnesium chloride, 0.001% poloxamer 188.").

247. Claim 26 of the '313 Patent depends from claim 20, and further recites "wherein the liquid chromatography is reverse phase chromatography." Sarepta's method uses reverse phase liquid chromatography. *See* Exhibit U at [0024] ("In some aspects, the liquid chromatography is a reverse phase liquid chromatography."); cl. 4 ("The method of claim 1, wherein the liquid chromatography is a reverse phase liquid chromatography.").

248. Claim 27 of the '313 Patent depends from claim 26, and further recites "wherein the reverse phase chromatography is C8 reverse phase chromatography." Sarepta's method uses C8 reverse chromatography. *See* Exhibit U at [0024] ("In some aspects, the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column."); cl. 5 ("The method of claim 4, wherein the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column.").

249. On information and belief, Defendants analyze Elevidys® by LC/MS intact protein analysis according to the method recited in claims 20, 26 and 27 of the '313 Patent at least during release testing prior to commercial sale. On information and belief, this testing is required for commercialization of Elevidys®. To the extent Defendants do not perform the methods as

specifically set forth in the '595 Application, on information and belief, Sarepta performs a substantially similar method that includes each and every step of at least the methods claimed in claims 20, 26 and 27 of the '313 Patent.

250. Defendants' use of the patented methods during the manufacture of Elevidys® as claimed in the '313 Patent prior to the expiration of the '313 Patent constitutes direct infringement under 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, of at least claims 20, 26 and 27 of the '313 Patent.

251. On information and belief, Sarepta performs each and every step of at least the methods claimed in claims 20, 26 and 27 of the '313 Patent as part of the manufacturing process of the Elevidys® pharmaceutical composition prior to releasing any batch of Elevidys® for commercial sale. On information and belief, performance of these methods is required for commercialization of Elevidys®.

252. Defendants jointly infringe the '313 Patent by contracting with third-party contract manufacturers to manufacture Elevidys® under the direction and control of Sarepta, and/or by forming a joint enterprise with manufacturers and testing companies for the manufacture, testing, and sale of Elevidys®. *See, e.g.*, Sarepta May 2024 10-Q at p. 22 ("We have adopted a hybrid development and manufacturing strategy in which we have built internal expertise relative to all aspects of AAV-based manufacturing . . . while closely partnering with experienced manufacturing partners to expedite development and commercialization of our gene therapy programs. We have secured manufacturing capacity at Thermo and Catalent to support our clinical and commercial manufacturing demand for ELEVIDYS and our LGMD programs."); Exhibit H, Catalent Jan. 5, 2023 Press Release at pp. 1-2 ("Catalent will be Sarepta's primary commercial manufacturing partner for this therapy [Elevidys®]."); Exhibit F, § 3.2.A Facilities Table (The Elevidys® drug

substance and drug product are manufactured in Catalent facilities in Harmans, MD and Baltimore, MD, respectively). On information and belief, Defendants condition receipt of contractual benefits by third parties upon manufacture of Elevidys®, and establish the manner and timing of third parties' performance.

253. On information and belief, at least as of May 1, 2025, Defendants have actively induced third parties to infringe one or more claims of the '313 Patent, including but not limited to claims 20, 26 and 27 under 35 U.S.C. § 271(b), by instructing and contracting with third parties to manufacture Elevidys® in accordance with the claimed methods, with knowledge of the '313 Patent and that the induced acts would constitute infringement.

254. Moreover, on information and belief, Defendants contribute to infringement of the '313 Patent, including but not limited to claims 20, 26 and 27 under 35 U.S.C. § 271(c) by supplying materials or apparatuses for use in practicing the patented method, such as the provision of engineered rAAV virions to manufacture the finished drug product, such materials or apparatuses having no substantially non-infringing uses, with knowledge of the '313 Patent and its infringement at least as of May 1, 2025.

255. Plaintiff has suffered damages, including pre-suit damages, as a result of Defendants' infringement of the '313 Patent.

256. On information and belief, Sarepta has continued to manufacture, use, import, sell, or offer to sell Elevidys® in the United States after becoming aware of the '313 Patent.

257. Given Sarepta's knowledge of the '313 Patent from the May 2025 letter from Genzyme, Sarepta's continued infringement of the '313 Patent by its manufacture, use, importation, sale, or offer to sell Elevidys®, or inducing others to manufacture, use, import, sell, or offer to sell Elevidys®, is deliberate and intentional.

258. In addition, given Sarepta's knowledge of the '313 Patent from the May 2025 letter from Genzyme, Sarepta also knew or should have known that its continued manufacture and sale of Elevidys® after gaining knowledge of the '313 Patent constituted an unjustifiably high risk of infringement of the '313 Patent.

259. On information and belief, Sarepta's continued sale of Elevidys® and its infringement of the '313 Patent has been and continues to be deliberate, intentional, egregious, willful, and in reckless disregard of the valid patent claims of the '313 Patent, and entitles Genzyme to enhanced damages and attorneys' fees under 35 U.S.C. §§ 284 and 285. Accordingly, Sarepta's infringement of the '313 Patent has been willful.

PRAYER FOR RELIEF

WHEREFORE, Plaintiff respectfully requests that the Court:

A. Enter judgment that Defendants have infringed the '542 Patent, the '721 Patent, the '894 Patent, the '326 Patent, the '377 Patent, the '880 Patent, and the '313 Patent;

B. Enter judgment that Defendants' infringement of the '542 Patent, the '721 Patent, the '894 Patent, '326 Patent, '377 Patent, the '880 Patent, and the '313 Patent is deliberate and willful;

C. Award damages adequate to compensate Plaintiff for Defendants' infringement, including increased damages up to three times the amount found or assessed, together with pre-judgment and post-judgment interest and costs, under 35 U.S.C. § 284;

D. Enter judgment that this case is exceptional and award Plaintiff its reasonable attorneys' fees, costs, and expenses, under 35 U.S.C. § 285; and

E. Award such other and further relief as this Court may deem just and proper.

DEMAND FOR JURY TRIAL

Plaintiff hereby demands a trial by jury as to all issues so triable.

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Exhibit A

US009051542B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 9,051,542 B2**
(45) **Date of Patent: Jun. 9, 2015**

(54) **COMPOSITIONS AND METHODS TO PREVENT AAV VECTOR AGGREGATION**

(75) Inventors: **John Fraser Wright**, Princeton, NJ (US); **Guang Qu**, Alameda, CA (US)

(73) Assignee: **Genzyme Corporation**, Framingham, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **12/661,553**

(22) Filed: **Mar. 19, 2010**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 11/141,996, filed on Jun. 1, 2005, now Pat. No. 7,704,721.

(60) Provisional application No. 60/575,997, filed on Jun. 1, 2004, provisional application No. 60/639,222, filed on Dec. 22, 2004.

(51) **Int. Cl.**
C12N 7/00 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 7/00*** (2013.01); *C12N 2750/14151* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(Continued)

Primary Examiner — Satyendra Singh
(74) *Attorney, Agent, or Firm* — Roberta L. Robins; Robins Law Group

(57) **ABSTRACT**

Compositions and methods are provided for preparation of concentrated stock solutions of AAV virions without aggregation. Formulations for AAV preparation and storage are high ionic strength solutions (e.g. μ~500 mM) that are nonetheless isotonic with the intended target tissue. This combination of high ionic strength and modest osmolarity is achieved using salts of high valency, such as sodium citrate. AAV stock solutions up to 6.4×10^{13} vg/mL are possible using the formulations of the invention, with no aggregation being observed even after ten freeze-thaw cycles. The surfactant Pluronic® F68 may be added at 0.001% to prevent losses of virions to surfaces during handling. Virion preparations can also be treated with nucleases to eliminate small nucleic acid strands on virions surfaces that exacerbate aggregation.

6 Claims, 3 Drawing Sheets

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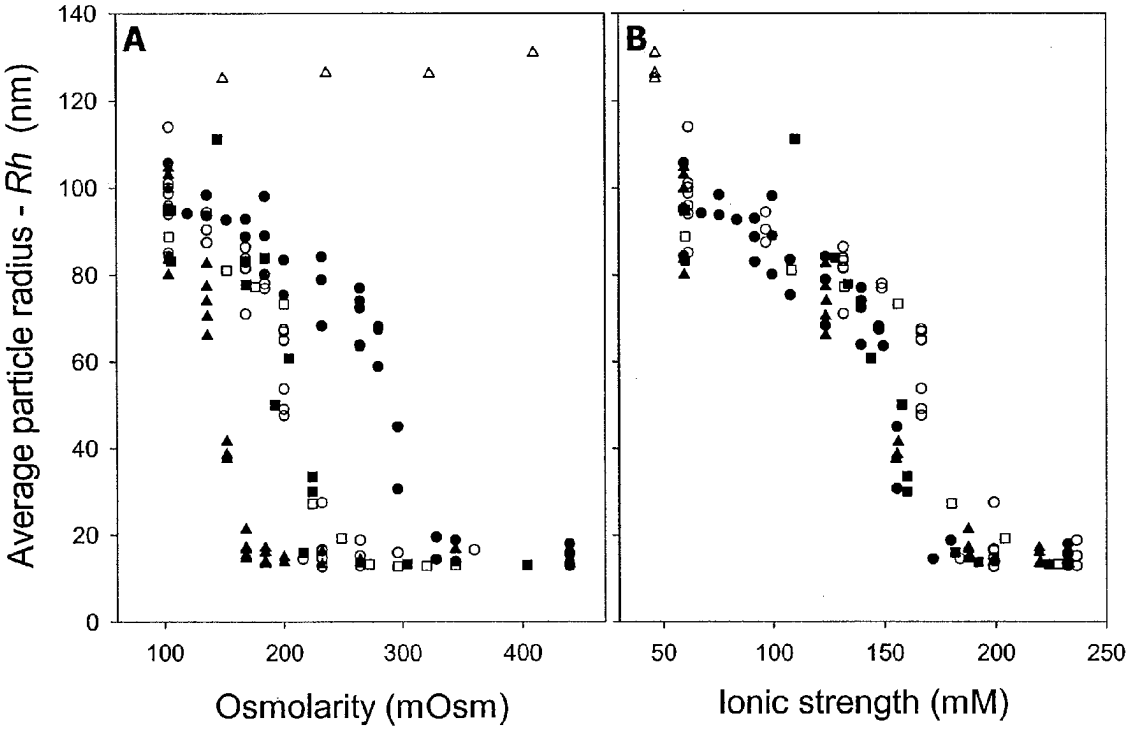
A
B
Average particle radius - Rh (nm)
140
120
100
80
60
40
20
0
100
200
300
400
Osmolarity (mOsm)
50
100
150
200
250
Ionic strength (mM)

FIGURE 1

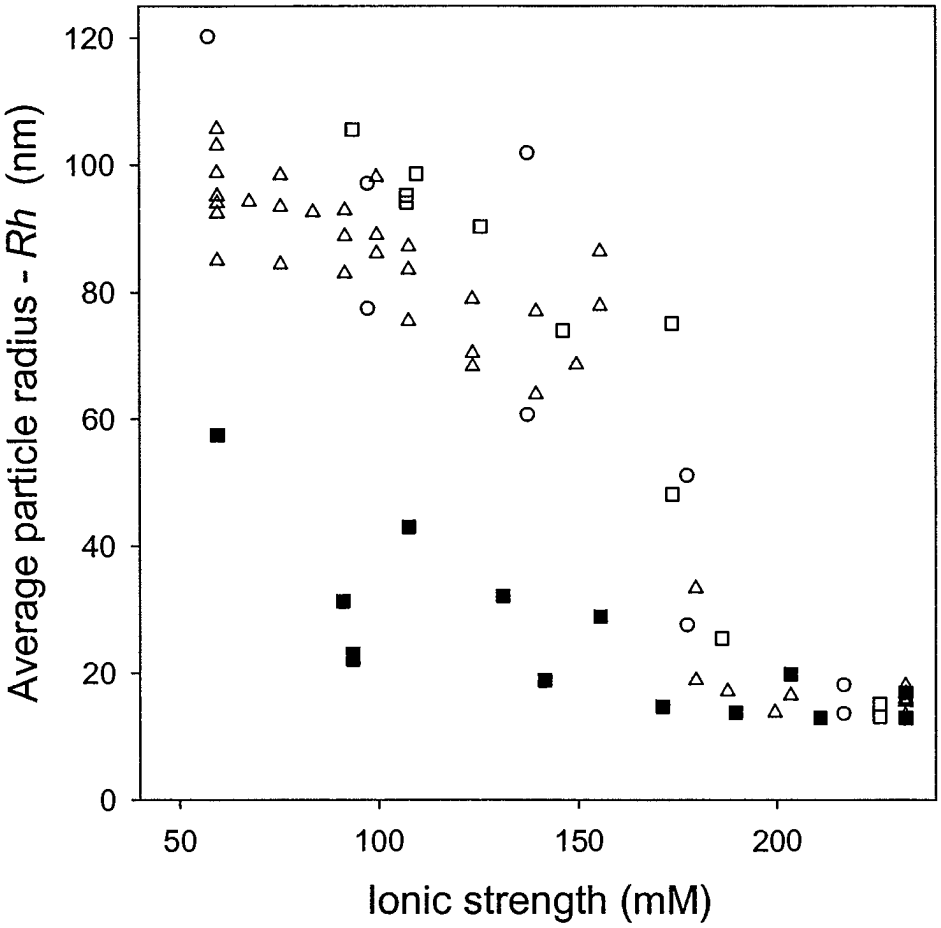
Average particle radius - Rh (nm)
120
100
80
60
40
20
0
50
100
150
200
Ionic strength (mM)

FIGURE 2

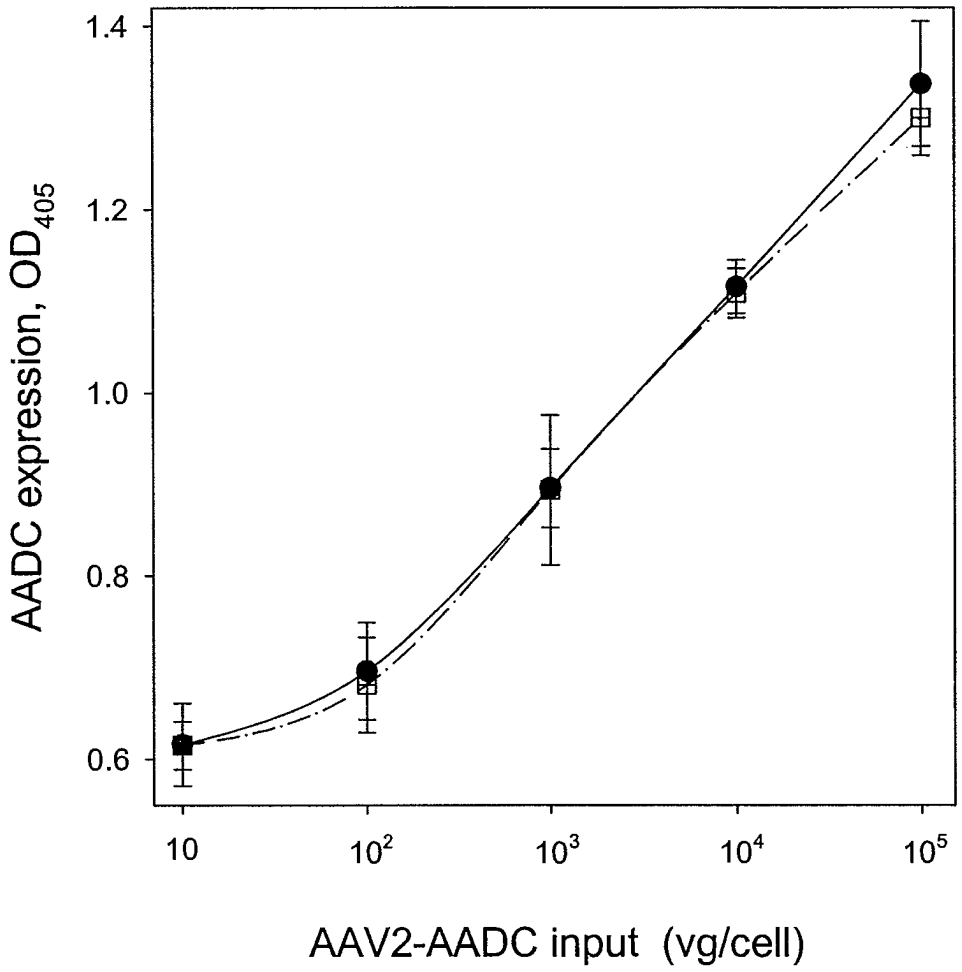
1.4
1.2
1.0
0.8
0.6
AADC expression, OD405
10
10^2
10^3
10^4
10^5
AAV2-AADC input (vg/cell)

FIGURE 3

COMPOSITIONS AND METHODS TO PREVENT AAV VECTOR AGGREGATION

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 11/141,996, now U.S. Pat. No. 7,704,721, from which application priority is claimed pursuant to 35 U.S.C. §120; which application claims the benefit under 35 U.S.C. §119(e) of provisional applications 60/575,997 filed Jun. 1, 2004 and 60/639,222 filed Dec. 22, 2004. The foregoing applications are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to compositions and methods of preparing and storing AAV virions that prevent aggregation.

BACKGROUND

Recombinant adeno-associated virus (rAAV) is a promising vector for human gene transfer. Grimm, D., and Kleinschmidt, J. A. (1999) *Hum Gene Ther.* 10: 2445-2450; High, K. A. (2001) *Ann. N.Y. Acad. Sci.* 953: 64-67; Pfeifer, A., and Verma, I. M. (2001) *Ann. Rev. Genomics Hum. Genet.* 2: 177-211. AAV is a member of the Dependovirus genus of the parvoviruses. AAV serotype 2 (AAV2) is composed of a single-strand DNA molecule of 4680 nucleotides encoding replication (rep) and encapsidation (cap) genes flanked by inverted terminal repeat (ITR) sequences. Berns, K. I. (1996) in *Fields Virology* (B. N. Fields et. al. Eds.), pp. 2173-2197. Lippincott-Raven Publishers, Philadelphia. The genome is packaged by three capsid proteins (VP1, VP2 and VP3), which are amino-terminal variants of the cap gene product. The resulting icosahedral virus particle has a diameter of ~26 nm. A high resolution crystal structure of AAV2 has been reported. Xie, Q. et al. (2002) *Proc. Natl. Acad. Sci. USA.* 99: 10405-10410.

The solubility of purified AAV2 virus particles is limited, and aggregation of AAV2 particles has been described as a problem. Croyle, M. A. et al. (2001) *Gene Therapy* 8: 1281-1290; Huang, J. et al. (2000) *Mol. Therapy* 1: 5286; Wright, J. F. et al. (2003) *Curr. Opin. Drug Disc. Dev.* 6: 174-178; Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27. In commonly used buffered-saline solutions, significant aggregation occurs at concentrations of 10^{13} particles/mL, and aggregation increases at higher concentrations. Huang and co-workers reported that AAV vectors undergo concentration-dependent aggregation. Huang, J. et al. (2000) *Mol. Therapy* 1: S286. Xie and coworkers (Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27) similarly reported that at concentrations exceeding 0.1 mg/mL, AAV2 vectors require elevated concentrations of salt to prevent aggregation. Aggregation of AAV2 vectors occurs at particle concentrations exceeding 10^{13} particles/mL in commonly used neutral-buffered solutions such as phosphate- and Tris-buffered saline. This corresponds to a protein concentration of ~0.06 mg/mL, and emphasizes the low solubility of AAV2 under these conditions. The effective vector concentration limit may be even lower for vectors purified using column chromatography techniques because excess empty capsids are co-purified and contribute to particle concentration.

Particle aggregation is a significant and not fully resolved issue for adenovirus vectors as well. Stability of a recently established adenovirus reference material (ARM) was recently reported. Adadevoh, K. et al. (2002) *BioProcessing* 1(2): 62-69. Aggregation of the reference material, formulated in 20 mM Tris, 25 mM NaCl, and 2.5% glycerol at pH 8.0, was assessed by dynamic light scattering, photon correlation spectroscopy and visual appearance. A variable level of vector aggregation following either freeze-thaw cycling or non-frozen storage was observed, resulting in restrictive protocols for the use of the ARM.

Aggregation can lead to losses during purification and inconsistencies in testing of purified vector preparations. The in vivo administration of AAV2 vectors to certain sites, such as the central nervous system, may require small volumes of highly concentrated vector, and the maximum achievable dose may be limited by low vector solubility.

Vector aggregation is also likely to influence biodistribution following in vivo administration, and cause adverse immune responses to vectors following their administration. As has been reported for proteins (Braun, A. et al. (1997) *Pharm. Res.* 14: 1472-1478), aggregation of vector may increase immunogenicity by targeting the vector to antigen presenting cells, and inducing enhanced immune responses to the capsid proteins and transgene product. The reports of immune responses to AAV vectors in pre-clinical (Chenuaud, P. et al. (2004) *Blood* 103: 3303-3304; Flotte, T. R. (2004) *Human Gene Ther.* 15: 716-717; Gao, G. et al. (2004) *Blood* 103: 3300-3302) and clinical (High, K. A. et al. (2004) *Blood* 104: 121a) studies illustrate the need to address all factors that may contribute to vector immunogenicity.

Testing protocols to characterize purified vectors are also likely to be affected by vector aggregation. Determination of the infectivity titer of vector was reported to be highly sensitive to vector aggregation. Zhen, Z. et al. (2004) *Human Gene Ther.* 15: 709-715. An important concern is that vector aggregates may have deleterious consequences following their in vivo administration because their transduction efficiency, biodistribution and immunogenicity may differ from monomeric particles. For example, intravascular delivery of AAV vectors to hepatocytes requires that the vectors pass through the fenestrated endothelial cell lining of hepatic sinusoids. These fenestrations have a radius ranging from 50 to 150 nm (Meijer, K. D. F., and Molema, G. (1995) *Sem. Liver Dis.* 15: 206) that is predicted to allow the passage of monomeric AAV vectors (diameter ~26 nm), but prevent the passage of larger vector aggregates. In biodistribution studies in mice, aggregated AAV2 vectors labeled with the fluorescent molecule Cy3 were sequestered in liver macrophages following vascular delivery. Huang, J. et al. (2000) *Mol. Therapy* 1: S286.

Formulation development for virus-based gene transfer vectors is a relatively recent area of investigation, and only a few studies have been reported describing systematic efforts to optimize AAV vector formulation and stability. Croyle, M. A. et al. (2001) *Gene Therapy* 8: 1281-1290; Wright, J. F. et al. (2003) *Curr. Opin. Drug Disc. Dev.* 6: 174-178; Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27. Defining formulations compatible with pre-clinical and clinical applications that minimize changes in vector preparations is an important requirement to achieve consistently high vector safety and functional characteristics. As is well established for protein therapeutics (Chen, B. et al. (1994) *J. Pharm. Sci.* 83: 1657-1661; Shire, S. J. et al. (2004) *J. Pharm. Sci.* 93: 1390-1402; Wang, W. (1999) *Int. J. Pharm.* 185: 129-188; Won, C. M. et al. (1998) *Int. J. Pharm.* 167: 25-36), an important aspect of vector stability is solubility during preparation and storage, and vector aggregation is a problem that needs to be fully addressed. Vector aggregation leads to losses during vector purification, and while aggregates can be removed by filtration, the loss in yield results in higher costs and capacity

limitations when producing vector for pre-clinical and clinical studies. Even after filtration to remove aggregates, new aggregates can form in concentrated preparations of AAV2 vector in buffered-saline solutions.

The need exists for improved formulations and methods for purification and storage of AAV vectors, such as rAAV2, that prevent aggregation of virus particles.

SUMMARY OF THE INVENTION

These and other needs in the art are met by the present invention, which provides high ionic strength solutions for use in preparing and storing AAV vectors that maintain high infectivity titer and transduction efficiency, even after freeze-thaw cycles.

In one aspect the invention relates to methods of preventing aggregation of virions in a preparation of virions by adding excipients to achieve an ionic strength high enough to prevent aggregation. In another aspect the invention relates to compositions of virions having an ionic strength high enough to prevent aggregation.

In some embodiments of the invention, the ionic strength is at least about 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, 600 mM, 700 mM or more. In some embodiments this ionic strength is accomplished using excipients comprising one or more multivalent ions, for example citrate, sulfate, magnesium or phosphate.

In additional embodiments, the osmolarity of the preparation of virions is maintained at near isotonic levels, for example 200 mOsm, 250 mOsm, 280 mOsm, 300 mOsm, 350 mOsm or 400 mOsm, even though the ionic strength is high enough to prevent virion aggregation.

In some embodiments the virions are adeno-associated virus (AAV) virions, for example AAV-2.

In other embodiments of the methods of the present invention preparations of virions are treated with a nuclease, for example Benzonase®. In further embodiments, nuclease treatment is combined with addition of excipients that achieve an ionic strength high enough to prevent aggregation.

In some embodiments of the present invention, the surfactant Pluronic® F68 is added to a preparation of virions, for example to 0.001%. In one embodiment, the composition comprises purified virus particles, 10 mM Tris pH 8.0, 100 mM sodium citrate and 0.001% Pluronic® F68.

In one embodiment, AAV vectors can be stored as compositions of the present invention at concentrations exceeding 1×10^{13} vg/mL, for example 2×10^{13}, 3×10^{13}, 4×10^{13}, 5×10^{13} and up to 6.4×10^{13} vg/mL, without significant aggregation. In some embodiments, AAV vectors stored using the methods and compositions of the invention do not exhibit significant aggregation when stored at 4° C. for five days. In other embodiments, AAV vectors that are stored as such compositions do not exhibit significant aggregation after one, five, ten or more freeze-thaw cycles at −20° C. or at −80° C.

In some embodiments, preparations of virions stored according to the methods and compositions of the invention exhibit an average particle radius (Rh), as measured by dynamic light scattering, indicating that no significant aggregation of virions has taken place. In some embodiments, preparations of virions stored according to the methods and compositions of the invention exhibit an average particle radius (Rh) greater than about 15 nm, 20 nm, or 30 nm.

In some embodiments, recovery of virions from preparations of virions stored according to the methods and compositions of the invention is greater than about 85%, 90% or 95% following filtration through a 0.22 μm filter.

In yet another aspect, the invention relates to kits comprising the high ionic strength formulations of the invention. In one embodiment the kit comprises a pre-mixed solution of excipients. In another embodiment the kit comprises two or more separate components of a high ionic strength composition of the present invention to be mixed by a user. In some embodiments the kit comprises sodium citrate, Tris® and Pluronic® F68. In other embodiments, the kit further comprises instructions for making a composition or performing a method of the present invention.

DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B present data showing aggregation of AAV2-FIX particles as a function of osmolarity (FIG. **1**A) or ionic strength (FIG. **1**B) for various buffer compositions. AAV2-FIX vectors are prepared by Method 2 of Example 1. Average particle radius is measured by dynamic light scattering (DLS) following vector dilution in varying concentrations of excipients buffered with 10 mM sodium phosphate at pH 7.5. Excipients include sodium chloride (●), sodium citrate (○), sodium phosphate (■), sodium sulfate (□), magnesium sulfate (▲), and glycerol (Δ).

FIG. **2** presents data on AAV2-FIX aggregation as a function of the method of purification. The average particle radius is measured by DLS following vector dilution in varying concentrations of sodium chloride buffered with 10 mM sodium phosphate at pH 7.5. Vectors are purified by Method 1 (double CsCl gradient) (○); Method 2 (cation exchange chromatography) (□); Method 2 plus nuclease digestion (■); or Method 3 (chromatography plus one CsCl gradient) (Δ). Purification Methods 1-3 are described in Example 1.

FIG. **3** presents data on transgene expression from D7/4 cells transduced with rAAV2-AADC virions prepared and stored in high ionic strength formulation (□) or in a control formulation (●). The concentration of AADC was measured by ELISA (in triplicate for each data point) 72 hours post-transduction. Error bars represent standard deviations.

DETAILED DESCRIPTION OF THE INVENTION

AAV2 vector aggregation is frequently observed in concentrated preparations of vectors and can affect purification recovery, and in vivo potency and safety. Hence, an important objective for the development AAV2 vectors is to identify methods and formulations that prevent aggregation of vectors when concentrated stocks are prepared.

Unless otherwise indicated, the term "vector" as used herein refers to a recombinant AAV virion, or virus particle, regardless of the frequent use of "vector" to also refer to non-viral DNA molecules, such as plasmids, in other contexts.

The present invention is based in part on the observation that solution ionic strength is an important parameter in AAV vector aggregation, implicating the involvement of ionic interactions between virus particles in the aggregation process. The observation that elevated ionic strength increases AAV2 vector solubility regardless of the identity of the charged excipient supports the hypothesis that ionic strength of solution per se, rather than interactions involving a specific ionic species, is the relevant physico-chemical parameter. A threshold ionic strength of at least 200 mM is required to prevent aggregation at vector particle concentrations examined herein.

Of practical concern, commonly used buffered saline solutions have insufficient ionic strength to prevent AAV2 vector aggregation at concentrations exceeding 10^{13} particles/mL. It

is known that high salt concentrations increase AAV2 vector solubility (e.g. highly concentrated AAV2 vectors recovered from gradients generally remain soluble in concentrated CsCl). However, optimal formulations for pre-clinical and clinical studies should be close to isotonic (280-400 mOsm), especially for in vivo administration of vector to sites where dilution of hypertonic solutions may be slow. In embodiments of the present invention the exponential relationship of ionic strength with charge valency is used to develop isotonic formulations with high ionic strengths. Salt species with multiple charge valencies (e.g. salts of sulfate, citrate, and phosphate) that are commonly used as excipients in human parenteral formulations can provide the level of ionic strength needed to prevent AAV2 vector aggregation when used at isotonic concentrations. While isotonic (150 mM) sodium chloride has an ionic strength of 150 mM, a value insufficient to maintain AAV2 solubility at high vector concentrations, isotonic sodium citrate, with an ionic strength of ~500 mM, can support AAV2 vector concentrations of at least 6.4×10^{13} vg/mL without aggregation.

Without intending to be limited by theory, the low solubility of AAV2 particles may be caused by their highly symmetrical nature in conjunction with the stabilizing effect of complementary charged regions between neighbouring particles in aggregates. The surface charge density based on the crystal structure of AAV2 (Xie, Q. et al. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99: 10405-10410) reveals a pattern of positive and negative charges on the virus surface. Previous reports have shown that AAV2 vector aggregation is pH dependent, and hypothesized that amino acids with charged side groups are involved in inter-particle binding. Qu, G. et al. (2003) *Mol. Therapy* 7: S238. These reports hypothesized that if charged amino acid side chains are involved in vector aggregation, high concentrations of free amino acids could block vector particle interactions. However, we have found that amino acids with charged side chains are not effective in preventing AAV2 vector aggregation beyond their contribution to ionic strength.

Vector aggregation at low ionic strength was also found to be reduced but not prevented by efficient nuclease treatment of purified vector particles. Digestion at an earlier stage of the purification process (clarified HEK cell lysate) did not reduce aggregation following vector purification. It is likely that digestion of already purified virions is more efficient because of a higher enzyme to nucleic acid substrate ratio. One mechanism to explain these results is that residual nucleic acid impurities (e.g. host cell and plasmid DNA) bound to the vector surface can bridge to binding sites on neighbouring virus particles and thus cause aggregation. Purified AAV2 vectors (empty capsid free) have been reported to contain approximately 1% non-vector DNA. Smith, P. et al. (2003) *Mol. Therapy* 7: 5348. While >50% of this non-vector DNA was reported to be nuclease resistant and was packaged within capsid particles, some impurity DNA was nuclease resistant and appeared to be associated with the surface of purified vector particles. The observation that efficient nuclease treatment can reduce vector aggregation suggests that nucleic acids associated with the vector surface at an average level not greater than ~25 nucleotides per vector particle can contribute to AAV vector aggregation.

In summary, the use of high ionic strength solutions during AAV2 vector purification and final formulation, and efficient removal of residual vector surface DNA are two effective strategies to achieve highly concentrated solutions of AAV2 vectors for use in pre-clinical and clinical studies. High ionic strength solutions and nuclease treatment can be used in combination or separately. Although data were obtained using AAV2 vectors, the composition and methods of the present invention may also be useful with other AAV serotypes/variants, or other viral vectors such as adenoviruses, lentiviruses and retroviruses.

AAV Aggregation as a Function of Excipient Concentration

Initial screening experiments are performed to elucidate the mechanism of AAV vector aggregation and to identify classes of excipients that can reduce/prevent aggregation. Vector aggregation can be caused by dilution (5-fold) of vector in neutral-buffered saline with low concentration buffer (20 mM sodium phosphate, pH 7.2). Excipients are screened using this "dilution-stress" method to identify excipients that are able to prevent vector aggregation when included in the diluent. For screening, aggregation is measured by dynamic light scattering (DLS). Classes of excipients examined included selected inorganic salts, amino acids, uncharged carbohydrates, and surfactants. Results are presented in Table 1.

TABLE 1

SCREENING FOR EXCIPIENTS THAT PREVENT AAV2 VECTOR AGGREGATION USING DILUTION-STRESS METHOD

Excipient	Osm required to prevent aggregation (max tested)
Magnesium sulfate	180 mOsm
Sodium citrate	220 mOsm
Sodium chloride	320 mOsm
Sodium phosphate	220 mOsm
Sodium sulfate	220 mOsm
Arginine	NIA (200 mOsm)
Aspartic acid	320 mOsm
Glutamic acid	320 mOsm
Glycine	NIA (200 mOsm)
Histidine	NIA (200 mOsm)
Lysine	300 mOsm
Glycerol	NIA (5% w/v, 543 mOsm)
Iodixanol	NIA (5% w/v, 32 mOsm)
Mannitol	NIA (5% w/v, 275 mOsm)
Sorbitol	NIA (5% w/v, 275 mOsm)
Sucrose	NIA (5% w/v, 146 mOsm)
Trehalose	NIA (5% w/v, 146 mOsm)
Pluronic ® F68	NIA (10% w/v, 12 mOsm)
Polysorbate 80	NIA (1% w/v)

NIA: No inhibition of aggregation

As illustrated in Table 1, charged excipients (inorganic salts and amino acids) prevent aggregation when present at sufficient concentrations. However, salt concentrations required to prevent vector aggregation vary, ranging from 180 mOsm for magnesium sulfate, to 320 mOsm for sodium chloride. The amino acids arginine, aspartic acid, glutamic acid, glycine, histidine, and lysine do not prevent aggregation at 200 mOsm, but lysine, aspartic acid, and glutamic acid prevent aggregation at 300-320 mOsm. Arginine, glycine and histidine were not tested at concentrations other than 200 mOsm. Selected carbohydrates have no effect on vector particle aggregation when present at concentrations up to 5% w/v. For example, 5% w/v glycerol (543 mOsm) does not prevent aggregation. The surfactants Polysorbate80 (1% w/v) and Pluronic® F68 (10% w/v) similarly have no effect on aggregation using the "dilution-stress" method.

AAV Aggregation as a Function of Osmolarity and Ionic Strength

FIGS. **1**A and **1**B show the results of a more detailed analysis of vector aggregation as a function of the concentration of various salts. FIG. **1**A shows vector aggregation as a function of the osmolarity of selected excipients. For charged species a concentration-dependent inhibition of AAV2 vector

aggregation is observed. Salts with multivalent ions achieve a similar degree of inhibition of aggregation at lower concentrations than monovalent sodium chloride. For example, magnesium sulfate prevents aggregation at >200 mOsm whereas sodium chloride requires ≥350 mOsm to achieve a similar effect. Sodium citrate, sodium sulfate, and sodium phosphate are intermediate in their potency to prevent vector aggregation.

Although the results in FIG. **1**A and Table 1 show no effect of glycerol and certain sugars at concentrations up to 5% on AAV2 vector aggregation induced by low ionic strength, the data cannot rule out improvement of AAV2 solubility at glycerol concentrations above 5%. For example, Xie and coworkers reported that 25% (w/v) glycerol enabled concentration of AAV2 to very high concentrations (4.4 to 18×10^{14} particles/ml) in low ionic strength solutions. Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27.

FIG. **1**B shows the data of FIG. **1**A plotted as a function of the calculated ionic strength, rather than osmolarity, for each excipient. FIG. **1**B demonstrates that vector aggregation is prevented when ionic strength is ~200 mM or greater regardless of which salt is used. These data suggested that the ionic strength (μ) of a solution, a parameter that depends on both solute concentration and charge valency, is the primary factor affecting aggregation.

Ionic strengths useful to prevent aggregation in embodiments of the present invention include, for example, 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, 600 mM, 700 mM or higher ionic strengths. Multivalent ions are preferred to achieve these ionic strengths in methods and formulations of the present invention, such as divalent, trivalent, tetravalent, pentavalent ions and ions of even higher valency. The pH buffer in solutions and formulations of the present invention may be phosphate, Tris, or HEPES (or other Good's buffers), but any other suitable pH buffer may be used. In preferred embodiments, the multivalent ions and buffer are selected to be compatible with the target tissue for the vector being prepared.

Use of multivalent ions in the methods and compositions of the invention makes it possible to create compositions of high ionic strength but relatively low osmolarity. High ionic strength compositions of the present invention may be nearly isotonic, and may be, for example, about 200 mOsm, 250 mOsm, 280 mOsm, 300 mOsm, 350 mOsm or 400 mOsm, although other osmolarities may be acceptable for some uses of the compositions.

AAV Aggregation as a Function of the Method of AAV Purification

Recombinant AAV2 purified using different methods (e.g. density gradient purification versus ion-exchange chromatography) would be expected to have different impurity profiles. FIG. **2** shows vector aggregation as a function of ionic strength for several preparations of AAV differing in the purification method. Purification methods are described in Example 1. Sodium chloride is used to vary the ionic strength. AAV2-FIX vectors purified by double cesium chloride gradient ultracentrifugation (Method 1), by cation exchange column chromatography (Method 2), or by combined column and cesium chloride gradient ultracentrifugation (Method 3) each demonstrate similar aggregation responses as ionic strength is decreased. In contrast, AAV2-FIX purified by the column method and then subjected to a nuclease digestion step (Method 2+nuclease) shows reduced aggregation at low ionic strength.

AAV Aggregation at Preparative Scale

The data in Table 1 and FIGS. **1**A, **1**B and **2** involve vector aggregation at an analytical scale, employing DLS to measure aggregation. Table 2, in contrast, shows the effects of elevated ionic strength and nuclease treatment on AAV2 vector aggregation at a larger scale, using methods to induce and quantify vector aggregation that are relevant to preparative scale vector purification. Experimental details are provided in Example 2. Purified AAV vectors are diafiltered into solutions of various ionic strengths, the volume is reduced to achieve high vector concentrations, and aggregation is then assessed by measuring vector recovery after filtration through a 0.22 μm filter. Aliquots from a single pool of AAV2-AADC vector purified by Method 1 through the second CsCl gradient centrifugation step (1.8×10^{15} vg in 91 mL, 1.8×10^{13} vg/mL, in ~3M CsCl) are used as starting material in the diafiltration experiments. Tangential flow filtration using hollow fibers is used for diafiltration because it is scalable and yet it still enables preparation of volumes (min. 1.4 mL), and thus AAV concentrations, at which aggregation would be expected in neutral buffered saline.

In Experiment 1, three hollow fiber units are used to diafilter AAV2-AADC vector in formulations CF, TF1, or TF2, and the volume is reduced to a target of 2.5×10^{13} vg/mL. See Example 2. The samples are then filtered through a 0.22 μm filter. Results are shown in Table 2. Vector recovery ("Yield %") for both elevated ionic strength formulations TF1 (95±7.4%) and TF2 (93±7.4%) are significantly higher than the recovery using the control formulation CF (77±6.6%).

TABLE 2

AAV VECTOR RECOVERY AT PROCESS SCALE

Experiment	Formulation	μ (mM)	Target (vg/mL)	Actual (vg/mL)	Yield % (RSD)
1	CF	160	2.5E13	1.93E13	77 (6.6)
1	TF1	310	2.5E13	2.38E13	95 (7.4)
1	TF2	510	2.5E13	2.33E13	93 (7.4)
2	CF	160	6.7E13	3.98E13	59 (6.0)
2	TF2	510	6.7E13	6.42E13	96 (4.4)
3	CF (−Bz)	160	3.6E13	2.46E13	68 (11)
3	CF (+Bz)	160	3.6E13	3.29E13	91 (12)

In Experiment 2, AAV2-AADC is concentrated to a higher target value (6.7×10^{1} vg/mL) in CF or TF2. Vector recovery using TF2 (96±4.4%) is again significantly higher than recovery using CF (59±6.0%). Within the variability of the assays used, vector was recovered fully at both target concentrations using TF2, indicating that aggregation was prevented. In contrast, significant aggregation was observed at both target concentrations using CF, and the extent of aggregation (i.e. loss following 0.22 μm filtration) was higher at the higher target vector concentration. In an additional experiment (not shown), 50 μL samples of AAV2 vector are taken following concentration but prior to the 0.22 μm filtration step of Experiment 2, and examined by light microscopy. Vector concentrated in CF contains obvious amounts of visible material (not shown), while no such material is seen in vector concentrated in TF2.

Experiment 3 examines the effect of prior nuclease digestion of purified vector on aggregation. In the absence of nuclease digestion recovery of AAV2-AADC in CF is 68±11%, similar to the recoveries in Experiments 1 and 2. In contrast, purified vector treated with nuclease and then concentrated in CF gives higher recovery (91±12%). These prep scale results reflect the same effect of nuclease digestion shown in FIG. **2** using the "dilution-stress" (analytical scale) method.

The results presented in Table 2 demonstrate that the methods and compositions of the present invention increase the

recovery of AAV vector recovery. For example, in various embodiments of the present invention, recovery is improved from less than about 80% to at least about 85%, 90%, 95% or more.

AAV Stability and Activity Following Storage or Freeze-Thaw Cycling

Croyle and coworkers reported a significant loss of titer of AAV and adenovirus following multiple freeze-thaw cycling in sodium phosphate buffer, and demonstrated that the better pH buffering provided by potassium phosphate during freeze-thaw cycling prevented titer loss. Croyle, M. A. et al. (2001) *Gene Therapy* 8: 1281-1290. Results of our freeze-thaw stability study using sodium phosphate support these findings. We find that while 150 mM sodium phosphate provides sufficient ionic strength to prevent aggregation during preparation and non-frozen storage of concentrated AAV2-AADC vector, even a single freeze-thaw cycle at −20 or −80° C. results in aggregation.

AAV stability after storage or freeze-thaw (F/T) cycling is assessed in buffers of the present invention as follows. The concentrated vectors prepared in CF, TF1, and TF2 (Table 2, Experiment 1) are subjected to a short stability study to investigate whether aggregation will occur during refrigerated storage, or following multiple freeze-thaw (F/T) cycles. Aggregation is assessed by DLS using undiluted samples, and Rh values >20 nm are deemed to indicate the occurrence of some level of aggregation.

TABLE 3

STABILITY OF AAV2 VECTORS

	Particle radius - Rh (nm)							
Formu-		4° C.	−20° C.			−80° C.		
lation	Pre	5 d	1 F/T	5 F/T	10 F/T	1 F/T	5 F/T	10 F/T
CF	14.5	27.0	22.4	56.1	94.5	20.6	57.5	141
TF1	13.8	16.3	TH	TH	TH	TH	TH	TH
TF2	13.8	14.4	14.2	14.0	14.1	13.8	21.3	50.9

Pre: DLS radius measured immediately following 0.2 μm filtration.
Vector concentrations (vg/mL): CF: 1.93E13, TF1: 2.38E13, TF2: 2.33E13.
TH: signal intensity is too high to measure because of extensive aggregation.

As shown in Table 3, AAV2-AADC vector prepared in CF shows some aggregation after 5 days of storage at 4° C., as well as following one or more F/T cycles at −20 or −80° C. For vector prepared in TF1, no aggregation occurs after 5 days at 4° C., but aggregation occurs following a single F/T cycle at −20 or −80° C. as indicated by a DLS signal intensity that is too high to measure. Visual inspection of these samples reveals slight cloudiness, which is consistent with aggregation. For vector prepared in TF2, no aggregation is observed at 4° C., or following up to 10 F/T cycles at −20° C. Some aggregation is observed following 5 and 10 F/T cycles at −80° C.

AAV activity after storage or F/T cycling in TF2 is assessed as follows. As described above, the high ionic strength, isotonic formulation TF2 effectively prevents vector aggregation during concentration and storage, and therefore represents a promising candidate for further study. An important question is whether preparation and storage of the vector in high ionic strength TF2 would adversely affect its functional activity. To assess this, assays are performed to measure the infectious titer and the transduction efficiency of vectors prepared and stored for an extended period of time in TF2.

For infectivity, a highly sensitive infectivity assay capable of detecting single infectious events is used. Zhen, Z. et al. (2004) *Human Gene Ther.* 15: 709-715. AAV2-AADC is prepared in TF2 at a concentration of 6.4×10^{13} vg/mL. After being stored for 45 days at 4° C. the preparation has a vector genome to infectious unit ratio (vg/IU) of 13, compared to a value of 16 vg/IU for the reference vector. This difference is not significant given the reported variability of this assay (RSD ~50%).

Transduction efficiency is assessed by measuring the expression of AADC protein by ELISA following transduction of D7/4 cells. FIG. **3** shows no significant difference between vector prepared in TF2 and the reference control for vector input ranging from 10 to 10^5 vg/cell. Together, these data indicate that preparation and storage of AAV2 vectors in high ionic strength TF2 does not have a deleterious effect on vector infectivity or transduction efficiency.

CONCLUSION

The effect of ionic strength (μ) on virus particle interactions is determined to elucidate the mechanism of vector aggregation. The ionic strength of neutral-buffered isotonic saline (μ=150 mM) is insufficient to prevent aggregation of AAV2 vectors purified by gradient ultracentrifugation or by cation exchange chromatography at concentrations exceeding ~10^{13} particles/mL. Inclusion of sugars (sorbitol, sucrose, mannitol, trehalose, glycerol) at concentrations up to 5% (w/v) or of surfactants Tween80® (1%) or Pluronic® F68 (10%) does not prevent aggregation of vector particles.

In contrast, vector particles remain soluble when elevated ionic strength solutions (μ>200 mM) are used during purification and for final vector formulation. Elevated ionic strength solutions using isotonic excipient concentrations for in vivo administration are prepared with salts of multivalent ions, including sodium citrate, sodium phosphate, and magnesium sulfate. An isotonic formulation containing 10 mM Tris, 100 mM sodium citrate, 0.001% Pluronic® F68, pH 8.0 (μ~500 mM) enables concentration of AAV2-AADC vectors to 6.4×10^{13} vg/mL with no aggregation observed during preparation and following ten freeze-thaw cycles at −20° C. See Table 3, below, and accompanying discussion. AAV2-AADC vectors prepared and stored for an extended period in elevated ionic strength formulation retain high infectivity titer (13 IU/vg) and transduction efficiency.

Nuclease treatment of purified AAV2 vectors reduces the degree of vector aggregation, implicating vector surface nucleic acid impurities in inter-particle interactions. Hence, purification methods to efficiently remove vector surface residual nucleic acids, coupled with the use of elevated ionic strength isotonic formulations, are useful methods to prevent AAV2 vector aggregation.

Example 1

AAV Purification Methods

AAV2 vectors expressing human coagulation factor IX (FIX) or human amino acid decarboxylase (AADC) are produced by triple transfection of HEK293 cells as previously described (Matsushita, T. et al. (1998) *Gene Therapy* 5: 938-945), with modifications. For the large scale preparations, cells are cultured and transfected in 850 mm^2 roller bottles (Corning). Vectors are purified by one of three methods.

In purification Method 1, modified from Matsushita, transfected HEK293 cells in roller bottles are collected by centrifugation (1000 g, 15 min), resuspended in 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2, and lysed by three freeze/thaw cycles (alternating an ethanol/dry ice bath

and a 37° C. water bath). The cell lysate is clarified by centrifugation (8,000 g, 15 min). The supernatant is then diluted to 200 mM NaCl by addition of 10 mM sodium phosphate, pH 7.2, and digested with Benzonase® (Merck, Purity Grade 1; 200 U/mL, 1 h, 37° C.). The lysate is adjusted to 25 mM $CaCl_2$ using a 1M stock solution, and incubated at 4° C. for one hour.

The mixture is centrifuged (8,000 g, 15 min), and the supernatant containing vector is collected. To precipitate virus from the clarified cell lysate, polyethylene glycol (PEG8000) is added to a final concentration of 8%, the mixture incubated at 4° C. for three hours, and then centrifuged (8,000 g, 15 min). The pellets containing vector are re-suspended with mixing in 0.15M NaCl, 50 mM Hepes, 25 mM EDTA, pH 8.0 and incubated at 4° C. for 16 hours. The resuspended material is pooled, and solid cesium chloride is added to a final density of 1.40 gm/ml. Vector is then banded by ultracentrifugation (SW28, 27,000 rpm, 24 h, 20° C.) using a Beckman model LE-80 centrifuge. The centrifugation tubes are fractionated, and densities from 1.38 to 1.42 gm/mL containing vector are pooled. This material is banded a second time by ultracentrifugation (NVT65 rotor, 65,000 rpm, 16 h, 20° C.), and fractions containing purified AAV2 vectors are pooled. To concentrate vector and to perform buffer exchange, vectors in concentrated cesium chloride solution are subjected to ultrafiltration/diafiltration (UF/DF) by tangential flow filtration as described below (Example 2).

In purification Method 2, cell harvests containing AAV are microfluidized and filtered sequentially through 0.65 and 0.22 μm filters (Sartorius). Virus is purified from the clarified cell lysates by cation exchange chromatography using Poros HS50 resin as previously described. U.S. Pat. No. 6,593,123. For the nuclease digestion described in FIG. **2**, column-purified vectors are incubated (4 h, RT) with 100 U/mL Benzonase and 10 U/mL DNAse I (RNAse free, Roche Diagnostics, Indianapolis, Ind.).

For purification Method 3, AAV2 vectors purified by cation exchange chromatography are subjected to an additional cesium chloride gradient ultracentrifugation step (SW28, 27,000 rpm, 20 h) to remove empty capsids prior to UF/DF.

Real time quantitative PCR (Q-PCR) is used to quantify AAV preparations as previously described. Sommer, J. M. et al. (2003) *Mol. Therapy* 7: 122-128. Vectors purified by each of the three methods are analyzed by SDS-PAGE/silver staining analysis, and in all cases VP1, VP2 and VP3 are present in the expected ratios, with the capsid proteins representing >95% of total proteins as determined by scanning densitometry. However, unlike gradient-purified AAV2 vectors purified using Methods 1 and 3, vectors purified by Method 2 (column chromatography) contain empty capsids, ranging from 3-10 empty capsids per vector genome.

Example 2

Ultrafiltration and Diafiltration to Detect AAV Aggregation

Disposable hollow fiber tangential flow filtration devices (Amersham BioSciences 8" Midgee, 100 kDa nominal pore size) are used to concentrate and diafilter AAV2 vectors purified by the methods described above, and for the UF/DF experiments described in Table 2. For all UF/DF procedures a volume of diafiltration buffer corresponding to 10× the product volume is used, and it is added in ~1 mL increments to approximate continuous diafiltration. Using this method, the calculated residual CsCl after diafiltration is <0.5 mM.

The following three formulations were used for UF/DF: Control Formulation (CF: 140 mM sodium chloride, 10 mM sodium phosphate, 5% sorbitol, pH 7.3); Test Formulation 1 (TF1: 150 mM sodium phosphate, pH7.5); and Test Formulation 2 (TF2: 100 mM sodium citrate, 10 mM Tris, pH8.0). For Experiment 1 shown in Table 2, diafiltration is performed at a volume corresponding to a vector concentration of 1×10^{13} vg/mL, and following diafiltration the volume is reduced to a value corresponding to 2.5×10^{13} vg/mL (assuming no vector loss).

For Experiment 2, diafiltration is performed at a volume corresponding to a 2×10^{13} vg/mL, and the volume is then reduced to a value corresponding to 6.7×10^{13} vg/mL.

For Experiment 3 (CF±Bz), AAV2-AADC (approximately 1.2×10^{14} vg) is first diafiltered into TF1 (a formulation compatible with nuclease activity) and then passed through a 0.22 μm filter. The titer of this material is determined, and the volume is adjusted to correspond to a concentration of 1×10^{13} vg/mL. To 10 mL of this material, $MgCl_2$ is added to a concentration of 2 mM, and then divided into two equal aliquots. One aliquot is incubated with Benzonase (200 U/mL, 4 h, RT), and the second is mock-incubated. Each aliquot is then diafiltered at a volume corresponding to a vector concentration 2×10^{13} vg/mL, and then concentrated to a 3.6×10^{13} vg/mL target. Following all UF/DF protocols, Pluronic® F-68 (BASF Corp., Mount Olive, N.J.) from a 1% stock is added to the vector product to a final concentration of 0.001%, and the solution is passed through a 0.22 μm syringe filter (Sartorius). All UF/DF procedures are performed in a laminar flow cabinet.

Example 3

Measurement of Vector Aggregation by Dynamic Light Scattering

Purified vectors are analyzed for aggregation by dynamic light scattering (DLS) using a Protein Solutions DynaPro 99 (λ=825.4 nm). Primary data (particle radius—Rh, average value measured over 30 cycles, 10 cycles/min) are used for all analyses reported. A "dilution-stress" method is used to assess the effect of varying excipients on vector aggregation. In this method, 80 μL of test diluent is added to 20 μL of vector solution with mixing in the actual cuvette used for DLS measurement, and data collection is initiated within 10 seconds of mixing. Prior to addition of test diluents, the Rh value for AAV2 vector preparations is measured and confirmed to be <15 nm to ensure that the starting material is monomeric. Samples that are not 100% monomeric are passed through a 0.22 μm syringe disc filter (Sartorius, low protein binding) to remove aggregates.

The osmolarity and ionic strength values given in FIGS. **1** and **2** are calculated using all excipients present in the mixture (i.e. weighted: test diluent (80%) and starting vector formulation (20%)). Osmolarity is calculated according to the equation: Osmolarity=Σc_i, where c_i is the molar concentration of each solute species. The ionic strength (μ) is calculated according to the equation: $\mu=\frac{1}{2}\Sigma c_i z_i^2$, where z_i is the charge on each species. In conditions that resulted in vector aggregation (e.g. low μ) a progressive increase in Rh is observed over the course of data collection. To validate the use of the average Rh measured over the 3 minute interval following dilution as a reliable measure of aggregation, the average rate of increase of Rh (ΔRh/Δt) over the same time interval is also determined (not shown). Analysis of ΔRh/Δt gives results concordant with those obtained using the average Rh value reported in FIGS. **1** and **2**.

Example 4

AAV Virion Infectivity

Infectivity of AAV2-AADC vectors is determined using a highly sensitive assay as previously described. Zhen, Z. et al. (2004) *Human Gene Ther.* 15: 709-715. Briefly, samples are serially diluted (10-fold dilutions, 10 replicates/dilution) and added to D7/4 cells (modified HeLa cells expressing AAV rep and cap) grown in 96 well tissue culture plates (Falcon, cat. #353227) in DMEM medium containing 10% FBS. Adenovirus (Ad-5, 100 vp/cell) is added to each well to provide helper functions. After 48 h, replication of AAV vector in each well is quantified by Q-PCR using transgene-specific primers and probes, and the frequency of infection at limiting dilution is analyzed by the Karber method to calculate the infectivity titer. The test sample is run concurrently with an AAV2-AADC reference previously prepared in CF and stored at −80° C.

The transduction efficiency of AAV2 vectors is quantified by a whole cell ELISA. D7/4 cells grown in 96 well plates are infected with 10-fold serial dilutions of the test sample and reference vector, corresponding to 10 to 10^5 vg/cell input (5 replicates/dilution). After 48 h, the culture medium is removed, and cells are washed twice with 200 μL PBS (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.2). Cells are then permeabilized and fixed by addition of 100 μL of PBS containing 0.5% Triton X-100 and 4% paraformaldehyde to each well (15 min). The fixing solution is removed, and the cells are washed twice with PBS containing 0.5% Triton X-100. Non-specific sites are blocked by adding PBS containing 3% bovine serum albumin (BSA) and 0.5% Triton X-100 (60 min).

After washing, cells are incubated for one hour with rabbit anti-AADC IgG antibody (Chemicon, AB136), and washed. Cells are then incubated for one hour with alkaline phosphatase-conjugated goat anti-rabbit IgG, and washed. Antibodies are diluted 1:1000 in PBS containing 1% BSA, 0.5% Triton X-100. Substrate (PNPP, Pierce, cat. #34047) is then added (1 mg/mL in 1× diethanolamine substrate buffer, Pierce, cat. #34064), and after incubation for 30 min the concentration of cleaved substrate is measured spectrophotometrically (λ=405 nm). Human AADC expression as a function of vector input is fitted using a spline curve (SigmaPlot). The AAV2-AADC reference vector is measured concurrently with the test sample.

While preferred illustrative embodiments of the present invention are described, it will be apparent to one skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is intended in the appended claims to cover all such changes and modifications that fall within the true spirit and scope of the invention.

All publications, patents and patent applications referred to herein are hereby incorporated by reference in their entireties.

We claim:

1. A composition for the storage of purified, recombinant adeno-associated virus (AAV) vector particles, comprising:

purified, recombinant AAV vector particles at a concentration exceeding 1×10^{13} vg/ml up to 6.4×10^{13} vg/ml;

a pH buffer, wherein the pH of the composition is between 7.5 and 8.0; and

excipients comprising one or more multivalent ions selected from the group consisting of citrate, sulfate, magnesium, and phosphate; wherein the ionic strength of the composition is greater than 200 mM, and wherein the purified AAV vector particles are stored in the composition without significant aggregation.

2. The composition of claim **1**, further comprising ethylene oxide/propylene oxide block copolymer Pluronic® F68.

3. The composition of claim **2**, wherein the Pluronic® F68 is present at a concentration of 0.001% (w/v).

4. The composition of claim **1**, wherein the pH buffer is 10 mM Tris, pH 8.0 and the excipients comprise 100 mM sodium citrate.

5. The composition of claim **1**, wherein the purified, recombinant AAV vector particles have an average particle radius (Rh) of less than about 20 nm as measured by dynamic light scattering.

6. The composition of claim **1**, wherein recovery of the purified, recombinant virus particles is at least about 90% following filtration of the composition of said AAV vector particles through a 0.22 μm filter.

* * * * *

Disclaimer

9,051,542 B2 - John Fraser Wright, Princeton, NJ (US); Guang Qu, Alameda, CA (US). COMPOSITIONS AND METHODS TO PREVENT AAV VECTOR AGGREGATION. Patent dated June 9, 2015. Disclaimer filed June 16, 2023, by the assignee, Genzyme Corporation.

I hereby disclaim the following complete claims 1 and 2, of said patent.

(Official Gazette, August 22, 2023)

Exhibit B

US007704721B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 7,704,721 B2**
(45) **Date of Patent: Apr. 27, 2010**

(54) **COMPOSITIONS AND METHODS TO PREVENT AAV VECTOR AGGREGATION**

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(73) Assignee: **Genzyme Corporation**, Framingham, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **11/141,996**

(22) Filed: **Jun. 1, 2005**

(65) **Prior Publication Data**

US 2006/0035364 A1 Feb. 16, 2006

Related U.S. Application Data

(60) Provisional application No. 60/575,997, filed on Jun. 1, 2004, provisional application No. 60/639,222, filed on Dec. 22, 2004.

(51) **Int. Cl.**
C12N 7/02 (2006.01)

(52) **U.S. Cl.** **435/239**

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

Compositions and methods are provided for preparation of concentrated stock solutions of AAV virions without aggregation. Formulations for AAV preparation and storage are high ionic strength solutions (e.g. μ~500 mM) that are nonetheless isotonic with the intended target tissue. This combination of high ionic strength and modest osmolarity is achieved using salts of high valency, such as sodium citrate. AAV stock solutions up to 6.4×10^{13} vg/mL are possible using the formulations of the invention, with no aggregation being observed even after ten freeze-thaw cycles. The surfactant Pluronic® F68 may be added at 0.001% to prevent losses of virions to surfaces during handling. Virion preparations can also be treated with nucleases to eliminate small nucleic acid strands on virions surfaces that exacerbate aggregation.

11 Claims, 3 Drawing Sheets

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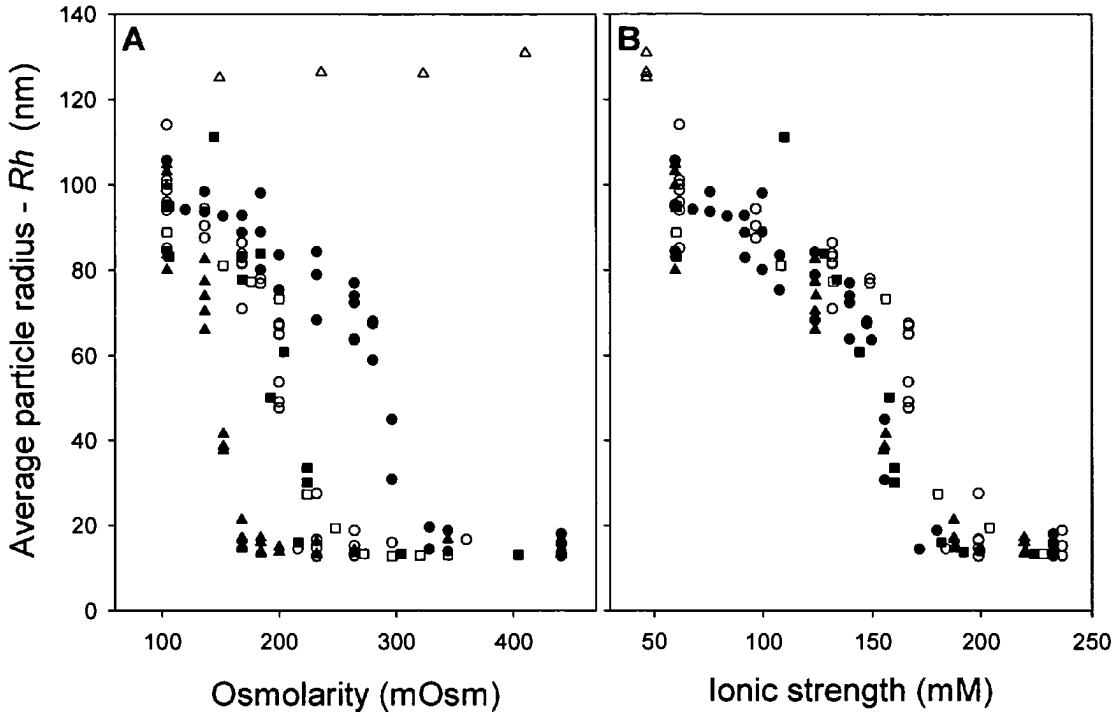
A
B
Average particle radius - Rh (nm)
0
20
40
60
80
100
120
140
100
200
300
400
Osmolarity (mOsm)
50
100
150
200
250
Ionic strength (mM)

FIGURE 1

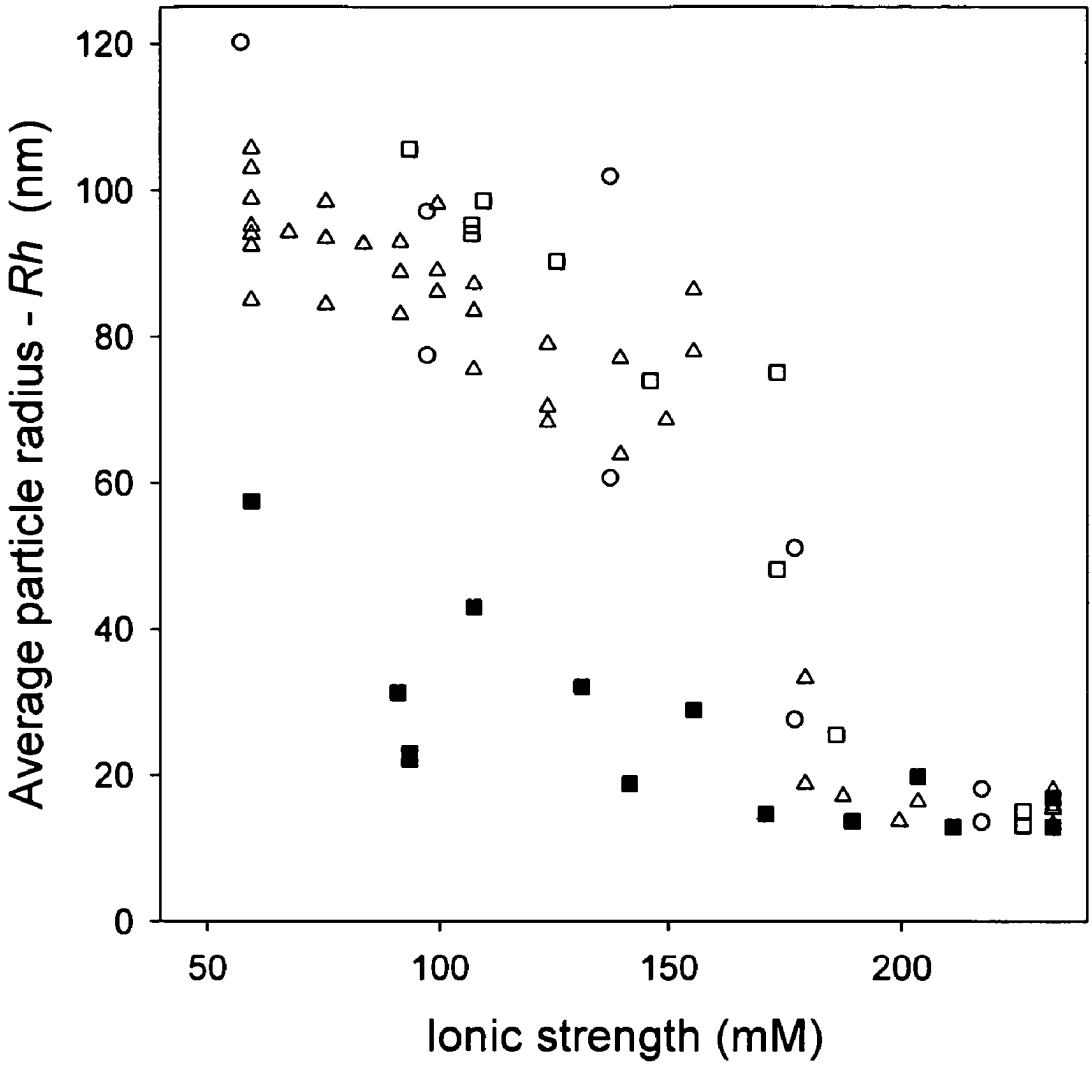
Average particle radius - Rh (nm)
120
100
80
60
40
20
0
50
100
150
200
Ionic strength (mM)

FIGURE 2

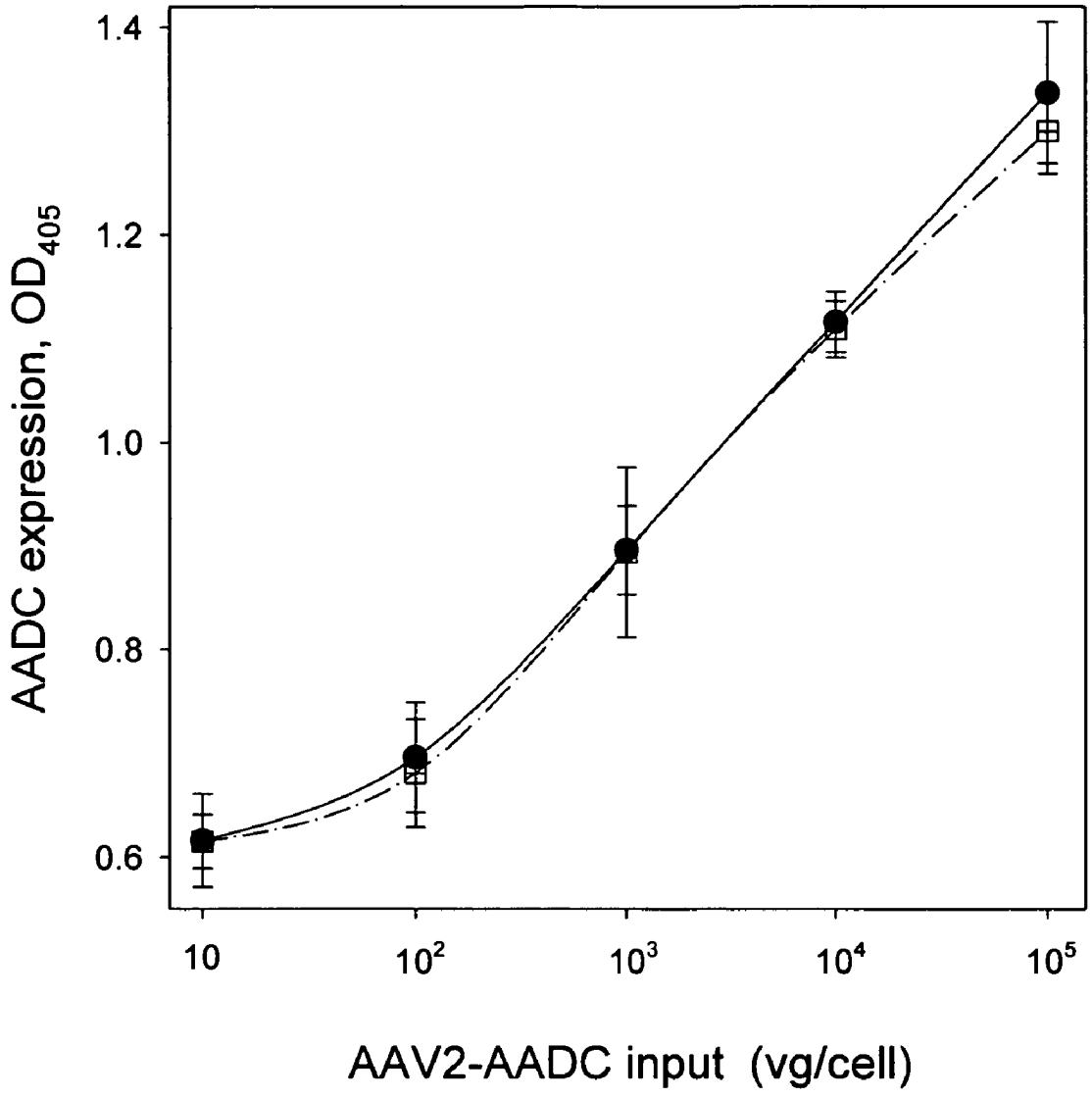
1.4
1.2
1.0
0.8
0.6
AADC expression, OD405
10
10^2
10^3
10^4
10^5
AAV2-AADC input (vg/cell)

FIGURE 3

COMPOSITIONS AND METHODS TO PREVENT AAV VECTOR AGGREGATION

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit under 35 U.S.C. § 119(e) of provisional applications 60/575,997 filed Jun. 1, 2004 and 60/639,222 filed Dec. 22, 2004, which applications are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to compositions and methods of preparing and storing AAV virions that prevent aggregation.

BACKGROUND

Recombinant adeno-associated virus (rAAV) is a promising vector for human gene transfer. Grimm, D., and Kleinschmidt, J. A. (1999) *Hum Gene Ther.* 10: 2445-2450; High, K. A. (2001) *Ann. N.Y. Acad. Sci.* 953: 64-67; Pfeifer, A., and Verma, I. M. (2001) *Ann. Rev. Genomics Hum. Genet.* 2: 177-211. AAV is a member of the Dependovirus genus of the parvoviruses. AAV serotype 2 (AAV2) is composed of a single-strand DNA molecule of 4680 nucleotides encoding replication (rep) and encapsidation (cap) genes flanked by inverted terminal repeat (ITR) sequences. Berns, K. I. (1996) in *Fields Virology* (B. N. Fields et. al. Eds.), pp. 2173-2197. Lippincott-Raven Publishers, Philadelphia. The genome is packaged by three capsid proteins (VP1, VP2 and VP3), which are amino-terminal variants of the cap gene product. The resulting icosahedral virus particle has a diameter of ~26 nm. A high resolution crystal structure of AAV2 has been reported. Xie, Q. et al. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99: 10405-10410.

The solubility of purified AAV2 virus particles is limited, and aggregation of AAV2 particles has been described as a problem. Croyle, M. A. et al. (2001) *Gene Therapy* 8: 1281-1290; Huang, J. et al. (2000) *Mol. Therapy* 1: S286; Wright, J. F. et al. (2003) *Curr. Opin. Drug Disc. Dev.* 6: 174-178; Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27. In commonly used buffered-saline solutions, significant aggregation occurs at concentrations of 10^{13} particles/mL, and aggregation increases at higher concentrations. Huang and co-workers reported that AAV vectors undergo concentration-dependent aggregation. Huang, J. et al. (2000) *Mol. Therapy* 1: S286. Xie and coworkers (Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27) similarly reported that at concentrations exceeding 0.1 mg/mL, AAV2 vectors require elevated concentrations of salt to prevent aggregation. Aggregation of AAV2 vectors occurs at particle concentrations exceeding 10^{13} particles/mL in commonly used neutral-buffered solutions such as phosphate- and Tris-buffered saline. This corresponds to a protein concentration of ~0.06 mg/mL, and emphasizes the low solubility of AAV2 under these conditions. The effective vector concentration limit may be even lower for vectors purified using column chromatography techniques because excess empty capsids are co-purified and contribute to particle concentration.

Particle aggregation is a significant and not fully resolved issue for adenovirus vectors as well. Stability of a recently established adenovirus reference material (ARM) was recently reported. Adadevoh, K. et al. (2002) *BioProcessing* 1(2): 62-69. Aggregation of the reference material, formulated in 20 mM Tris, 25 mM NaCl, and 2.5% glycerol at pH 8.0, was assessed by dynamic light scattering, photon correlation spectroscopy and visual appearance. A variable level of vector aggregation following either freeze-thaw cycling or non-frozen storage was observed, resulting in restrictive protocols for the use of the ARM.

Aggregation can lead to losses during purification and inconsistencies in testing of purified vector preparations. The in vivo administration of AAV2 vectors to certain sites, such as the central nervous system, may require small volumes of highly concentrated vector, and the maximum achievable dose may be limited by low vector solubility.

Vector aggregation is also likely to influence biodistribution following in vivo administration, and cause adverse immune responses to vectors following their administration. As has been reported for proteins (Braun, A. et al. (1997) *Pharm. Res.* 14: 1472-1478), aggregation of vector may increase immunogenicity by targeting the vector to antigen presenting cells, and inducing enhanced immune responses to the capsid proteins and transgene product. The reports of immune responses to AAV vectors in pre-clinical (Chenuaud, P. et al. (2004) *Blood* 103: 3303-3304; Flotte, T. R. (2004) *Human Gene Ther.* 15: 716-717; Gao, G. et al. (2004) *Blood* 103: 3300-3302) and clinical (High, K. A. et al. (2004) *Blood* 104: 121a) studies illustrate the need to address all factors that may contribute to vector immunogenicity.

Testing protocols to characterize purified vectors are also likely to be affected by vector aggregation. Determination of the infectivity titer of vector was reported to be highly sensitive to vector aggregation. Zhen, Z. et al. (2004) *Human Gene Ther.* 15: 709-715. An important concern is that vector aggregates may have deleterious consequences following their in vivo administration because their transduction efficiency, biodistribution and immunogenicity may differ from monomeric particles. For example, intravascular delivery of AAV vectors to hepatocytes requires that the vectors pass through the fenestrated endothelial cell lining of hepatic sinusoids. These fenestrations have a radius ranging from 50 to 150 nm (Meijer, K. D. F., and Molema, G. (1995) *Sem. Liver Dis.* 15: 206) that is predicted to allow the passage of monomeric AAV vectors (diameter~26 nm), but prevent the passage of larger vector aggregates. In biodistribution studies in mice, aggregated AAV2 vectors labeled with the fluorescent molecule Cy3 were sequestered in liver macrophages following vascular delivery. Huang, J. et al. (2000) *Mol. Therapy* 1: S286.

Formulation development for virus-based gene transfer vectors is a relatively recent area of investigation, and only a few studies have been reported describing systematic efforts to optimize AAV vector formulation and stability. Croyle, M. A. et al. (2001) *Gene Therapy* 8: 1281-1290; Wright, J. F. et al. (2003) *Curr. Opin. Drug Disc. Dev.* 6: 174-178; Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27. Defining formulations compatible with pre-clinical and clinical applications that minimize changes in vector preparations is an important requirement to achieve consistently high vector safety and functional characteristics. As is well established for protein therapeutics (Chen, B. et al. (1994) *J. Pharm. Sci.* 83: 1657-1661; Shire, S. J. et al. (2004) *J. Pharm. Sci.* 93: 1390-1402; Wang, W. (1999) *Int. J. Pharm.* 185: 129-188; Won, C. M. et al. (1998) *Int. J. Pharm.* 167: 25-36), an important aspect of vector stability is solubility during preparation and storage, and vector aggregation is a problem that needs to be fully addressed. Vector aggregation leads to losses during vector purification, and while aggregates can be removed by filtration, the loss in yield results in higher costs and capacity limitations when producing vector for pre-clinical and clinical studies. Even after filtration to remove aggregates, new

aggregates can form in concentrated preparations of AAV2 vector in buffered-saline solutions.

The need exists for improved formulations and methods for purification and storage of AAV vectors, such as rAAV2, that prevent aggregation of virus particles.

SUMMARY OF THE INVENTION

These and other needs in the art are met by the present invention, which provides high ionic strength solutions for use in preparing and storing AAV vectors that maintain high infectivity titer and transduction efficiency, even after freeze-thaw cycles.

In one aspect the invention relates to methods of preventing aggregation of virions in a preparation of virions by adding excipients to achieve an ionic strength high enough to prevent aggregation. In another aspect the invention relates to compositions of virions having an ionic strength high enough to prevent aggregation.

In some embodiments of the invention, the ionic strength is at least about 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, 600 mM, 700 mM or more. In some embodiments this ionic strength is accomplished using excipients comprising one or more multivalent ions, for example citrate, sulfate, magnesium or phosphate.

In additional embodiments, the osmolarity of the preparation of virions is maintained at near isotonic levels, for example 200 mOsm, 250 mOsm, 280 mOsm, 300 mOsm, 350 mOsm or 400 mOsm, even though the ionic strength is high enough to prevent virion aggregation.

In some embodiments the virions are adeno-associated virus (AAV) virions, for example AAV-2.

In other embodiments of the methods of the present invention preparations of virions are treated with a nuclease, for example Benzonase®. In further embodiments, nuclease treatment is combined with addition of excipients that achieve an ionic strength high enough to prevent aggregation.

In some embodiments of the present invention, the surfactant Pluronic® F68 is added to a preparation of virions, for example to 0.001%. In one embodiment, the composition comprises purified virus particles, 10 mM Tris pH 8.0, 100 mM sodium citrate and 0.001% Pluronic® F68.

In one embodiment, AAV vectors can be stored as compositions of the present invention at concentrations exceeding 1×10^{13} vg/mL, for example 2×10^{13}, 3×10^{13}, 4×10^{13}, 5×10^{13} and up to 6.4×10^{13} vg/mL, without significant aggregation. In some embodiments, AAV vectors stored using the methods and compositions of the invention do not exhibit significant aggregation when stored at 4° C. for five days. In other embodiments, AAV vectors that are stored as such compositions do not exhibit significant aggregation after one, five, ten or more freeze-thaw cycles at −20° C. or at −80° C.

In some embodiments, preparations of virions stored according to the methods and compositions of the invention exhibit an average particle radius (Rh), as measured by dynamic light scattering, indicating that no significant aggregation of virions has taken place. In some embodiments, preparations of virions stored according to the methods and compositions of the invention exhibit an average particle radius (Rh) greater than about 15 nm, 20 nm, or 30 nm.

In some embodiments, recovery of virions from preparations of virions stored according to the methods and compositions of the invention is greater than about 85%, 90% or 95% following filtration through a 0.22 μm filter.

In yet another aspect, the invention relates to kits comprising the high ionic strength formulations of the invention. In one embodiment the kit comprises a pre-mixed solution of excipients. In another embodiment the kit comprises two or more separate components of a high ionic strength composition of the present invention to be mixed by a user. In some embodiments the kit comprises sodium citrate, Tris® and Pluronic® F68. In other embodiments, the kit further comprises instructions for making a composition or performing a method of the present invention.

DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B present data showing aggregation of AAV2-FIX particles as a function of osmolarity (FIG. **1**A) or ionic strength (FIG. **1**B) for various buffer compositions. AAV2-FIX vectors are prepared by Method 2 of Example 1. Average particle radius is measured by dynamic light scattering (DLS) following vector dilution in varying concentrations of excipients buffered with 10 mM sodium phosphate at pH 7.5. Excipients include sodium chloride (●), sodium citrate (○), sodium phosphate (■), sodium sulfate (□), magnesium sulfate (▲), and glycerol (Δ).

FIG. **2** presents data on AAV2-FIX aggregation as a function of the method of purification. The average particle radius is measured by DLS following vector dilution in varying concentrations of sodium chloride buffered with 10 mM sodium phosphate at pH 7.5. Vectors are purified by Method 1 (double CsCl gradient) (○); Method 2 (cation exchange chromatography) (□); Method 2 plus nuclease digestion (■); or Method 3 (chromatography plus one CsCl gradient) (Δ). Purification Methods 1-3 are described in Example 1.

FIG. **3** presents data on transgene expression from D7/4 cells transduced with rAAV2-AADC virions prepared and stored in high ionic strength formulation (□) or in a control formulation (●). The concentration of AADC was measured by ELISA (in triplicate for each data point) 72 hours post-transduction. Error bars represent standard deviations.

DETAILED DESCRIPTION OF THE INVENTION

AAV2 vector aggregation is frequently observed in concentrated preparations of vectors and can affect purification recovery, and in vivo potency and safety. Hence, an important objective for the development AAV2 vectors is to identify methods and formulations that prevent aggregation of vectors when concentrated stocks are prepared.

Unless otherwise indicated, the term "vector" as used herein refers to a recombinant AAV virion, or virus particle, regardless of the frequent use of "vector" to also refer to non-viral DNA molecules, such as plasmids, in other contexts.

The present invention is based in part on the observation that solution ionic strength is an important parameter in AAV vector aggregation, implicating the involvement of ionic interactions between virus particles in the aggregation process. The observation that elevated ionic strength increases AAV2 vector solubility regardless of the identity of the charged excipient supports the hypothesis that ionic strength of solution per se, rather than interactions involving a specific ionic species, is the relevant physico-chemical parameter. A threshold ionic strength of at least 200 mM is required to prevent aggregation at vector particle concentrations examined herein.

Of practical concern, commonly used buffered saline solutions have insufficient ionic strength to prevent AAV2 vector aggregation at concentrations exceeding 10^{13} particles/mL. It is known that high salt concentrations increase AAV2 vector solubility (e.g. highly concentrated AAV2 vectors recovered from gradients generally remain soluble in concentrated

CsCl). However, optimal formulations for pre-clinical and clinical studies should be close to isotonic (280-400 mOsm), especially for in vivo administration of vector to sites where dilution of hypertonic solutions may be slow. In embodiments of the present invention the exponential relationship of ionic strength with charge valency is used to develop isotonic formulations with high ionic strengths. Salt species with multiple charge valencies (e.g. salts of sulfate, citrate, and phosphate) that are commonly used as excipients in human parenteral formulations can provide the level of ionic strength needed to prevent AAV2 vector aggregation when used at isotonic concentrations. While isotonic (150 mM) sodium chloride has an ionic strength of 150 mM, a value insufficient to maintain AAV2 solubility at high vector concentrations, isotonic sodium citrate, with an ionic strength of ~500 mM, can support AAV2 vector concentrations of at least 6.4×10^{13} vg/mL without aggregation.

Without intending to be limited by theory, the low solubility of AAV2 particles may be caused by their highly symmetrical nature in conjunction with the stabilizing effect of complementary charged regions between neighbouring particles in aggregates. The surface charge density based on the crystal structure of AAV2 (Xie, Q. et al. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99: 10405-10410) reveals a pattern of positive and negative charges on the virus surface. Previous reports have shown that AAV2 vector aggregation is pH dependent, and hypothesized that amino acids with charged side groups are involved in inter-particle binding. Qu, G. et al. (2003) *Mol. Therapy* 7: S238. These reports hypothesized that if charged amino acid side chains are involved in vector aggregation, high concentrations of free amino acids could block vector particle interactions. However, we have found that amino acids with charged side chains are not effective in preventing AAV2 vector aggregation beyond their contribution to ionic strength.

Vector aggregation at low ionic strength was also found to be reduced but not prevented by efficient nuclease treatment of purified vector particles. Digestion at an earlier stage of the purification process (clarified HEK cell lysate) did not reduce aggregation following vector purification. It is likely that digestion of already purified virions is more efficient because of a higher enzyme to nucleic acid substrate ratio. One mechanism to explain these results is that residual nucleic acid impurities (e.g. host cell and plasmid DNA) bound to the vector surface can bridge to binding sites on neighbouring virus particles and thus cause aggregation. Purified AAV2 vectors (empty capsid free) have been reported to contain approximately 1% non-vector DNA. Smith, P. et al. (2003) *Mol. Therapy* 7: S348. While >50% of this non-vector DNA was reported to be nuclease resistant and was packaged within capsid particles, some impurity DNA was nuclease resistant and appeared to be associated with the surface of purified vector particles. The observation that efficient nuclease treatment can reduce vector aggregation suggests that nucleic acids associated with the vector surface at an average level not greater than ~25 nucleotides per vector particle can contribute to AAV vector aggregation.

In summary, the use of high ionic strength solutions during AAV2 vector purification and final formulation, and efficient removal of residual vector surface DNA are two effective strategies to achieve highly concentrated solutions of AAV2 vectors for use in pre-clinical and clinical studies. High ionic strength solutions and nuclease treatment can be used in combination or separately. Although data were obtained using AAV2 vectors, the composition and methods of the present invention may also be useful with other AAV serotypes/variants, or other viral vectors such as adenoviruses, lentiviruses and retroviruses.

AAV Aggregation as a Function of Excipient Concentration

Initial screening experiments are performed to elucidate the mechanism of AAV vector aggregation and to identify classes of excipients that can reduce/prevent aggregation. Vector aggregation can be caused by dilution (5-fold) of vector in neutral-buffered saline with low concentration buffer (20 mM sodium phosphate, pH 7.2). Excipients are screened using this "dilution-stress" method to identify excipients that are able to prevent vector aggregation when included in the diluent. For screening, aggregation is measured by dynamic light scattering (DLS). Classes of excipients examined included selected inorganic salts, amino acids, uncharged carbohydrates, and surfactants. Results are presented in Table 1.

TABLE 1

SCREENING FOR EXCIPIENTS THAT PREVENT AAV2 VECTOR AGGREGATION USING DILUTION-STRESS METHOD

Excipient	Osm required to prevent aggregation (max tested)
Magnesium sulfate	180 mOsm
Sodium citrate	220 mOsm
Sodium chloride	320 mOsm
Sodium phosphate	220 mOsm
Sodium sulfate	220 mOsm
Arginine	NIA (200 mOsm)
Aspartic acid	320 mOsm
Glutamic acid	320 mOsm
Glycine	NIA (200 mOsm)
Histidine	NIA (200 mOsm)
Lysine	300 mOsm
Glycerol	NIA (5% w/v, 543 mOsm)
Iodixanol	NIA (5% w/v, 32 mOsm)
Mannitol	NIA (5% w/v, 275 mOsm)
Sorbitol	NIA (5% w/v, 275 mOsm)
Sucrose	NIA (5% w/v, 146 mOsm)
Trehalose	NIA (5% w/v, 146 mOsm)
Pluronic ® F68	NIA (10% w/v, 12 mOsm)
Polysorbate 80	NIA (1% w/v)

NIA: No inhibition of aggregation

As illustrated in Table 1, charged excipients (inorganic salts and amino acids) prevent aggregation when present at sufficient concentrations. However, salt concentrations required to prevent vector aggregation vary, ranging from 180 mOsm for magnesium sulfate, to 320 mOsm for sodium chloride. The amino acids arginine, aspartic acid, glutamic acid, glycine, histidine, and lysine do not prevent aggregation at 200 mOsm, but lysine, aspartic acid, and glutamic acid prevent aggregation at 300-320 mOsm. Arginine, glycine and histidine were not tested at concentrations other than 200 mOsm. Selected carbohydrates have no effect on vector particle aggregation when present at concentrations up to 5% w/v. For example, 5% w/v glycerol (543 mOsm) does not prevent aggregation. The surfactants Polysorbate80 (1% w/v) and Pluronic® F68 (10% w/v) similarly have no effect on aggregation using the "dilution-stress" method.

AAV Aggregation as a Function of Osmolarity and Ionic Strength

FIGS. **1**A and **1**B show the results of a more detailed analysis of vector aggregation as a function of the concentration of various salts. FIG. **1**A shows vector aggregation as a function of the osmolarity of selected excipients. For charged species a concentration-dependent inhibition of AAV2 vector aggregation is observed. Salts with multivalent ions achieve a

similar degree of inhibition of aggregation at lower concentrations than monovalent sodium chloride. For example, magnesium sulfate prevents aggregation at ≧200 mOsm whereas sodium chloride requires ≧350 mOsm to achieve a similar effect. Sodium citrate, sodium sulfate, and sodium phosphate are intermediate in their potency to prevent vector aggregation.

Although the results in FIG. **1**A and Table 1 show no effect of glycerol and certain sugars at concentrations up to 5% on AAV2 vector aggregation induced by low ionic strength, the data cannot rule out improvement of AAV2 solubility at glycerol concentrations above 5%. For example, Xie and coworkers reported that 25% (w/v) glycerol enabled concentration of AAV2 to very high concentrations (4.4 to 18×10^{14} particles/ml) in low ionic strength solutions. Xie, Q. et al. (2004) *J. Virol. Methods* 122: 17-27.

FIG. **1**B shows the data of FIG. **1**A plotted as a function of the calculated ionic strength, rather than osmolarity, for each excipient. FIG. **1**B demonstrates that vector aggregation is prevented when ionic strength is ~200 mM or greater regardless of which salt is used. These data suggested that the ionic strength (μ) of a solution, a parameter that depends on both solute concentration and charge valency, is the primary factor affecting aggregation.

Ionic strengths useful to prevent aggregation in embodiments of the present invention include, for example, 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, 600 mM, 700 mM or higher ionic strengths. Multivalent ions are preferred to achieve these ionic strengths in methods and formulations of the present invention, such as divalent, trivalent, tetravalent, pentavalent ions and ions of even higher valency. The pH buffer in solutions and formulations of the present invention may be phosphate, Tris, or HEPES (or other Good's buffers), but any other suitable pH buffer may be used. In preferred embodiments, the multivalent ions and buffer are selected to be compatible with the target tissue for the vector being prepared.

Use of multivalent ions in the methods and compositions of the invention makes it possible to create compositions of high ionic strength but relatively low osmolarity. High ionic strength compositions of the present invention may be nearly isotonic, and may be, for example, about 200 mOsm, 250 mOsm, 280 mOsm, 300 mOsm, 350 mOsm or 400 mOsm, although other osmolarities may be acceptable for some uses of the compositions.

AAV Aggregation as a Function of the Method of AAV Purification

Recombinant AAV2 purified using different methods (e.g. density gradient purification versus ion-exchange chromatography) would be expected to have different impurity profiles. FIG. **2** shows vector aggregation as a function of ionic strength for several preparations of AAV differing in the purification method. Purification methods are described in Example 1. Sodium chloride is used to vary the ionic strength. AAV2-FIX vectors purified by double cesium chloride gradient ultracentrifugation (Method 1), by cation exchange column chromatography (Method 2), or by combined column and cesium chloride gradient ultracentrifugation (Method 3) each demonstrate similar aggregation responses as ionic strength is decreased. In contrast, AAV2-FIX purified by the column method and then subjected to a nuclease digestion step (Method 2+nuclease) shows reduced aggregation at low ionic strength.

AAV Aggregation at Preparative Scale

The data in Table 1 and FIGS. **1**A, **1**B and **2** involve vector aggregation at an analytical scale, employing DLS to measure aggregation. Table 2, in contrast, shows the effects of elevated ionic strength and nuclease treatment on AAV2 vector aggregation at a larger scale, using methods to induce and quantify vector aggregation that are relevant to preparative scale vector purification. Experimental details are provided in Example 2. Purified AAV vectors are diafiltered into solutions of various ionic strengths, the volume is reduced to achieve high vector concentrations, and aggregation is then assessed by measuring vector recovery after filtration through a 0.22 μm filter. Aliquots from a single pool of AAV2-AADC vector purified by Method 1 through the second CsCl gradient centrifugation step (1.8×10^{15} vg in 91 mL, 1.8×10^{13} vg/mL, in ~3M CsCl) are used as starting material in the diafiltration experiments. Tangential flow filtration using hollow fibers is used for diafiltration because it is scalable and yet it still enables preparation of volumes (min. 1.4 mL), and thus AAV concentrations, at which aggregation would be expected in neutral buffered saline.

In Experiment 1, three hollow fiber units are used to diafilter AAV2-AADC vector in formulations CF, TF1, or TF2, and the volume is reduced to a target of 2.5×10^{13} vg/mL. See Example 2. The samples are then filtered through a 0.22 μm filter. Results are shown in Table 2. Vector recovery ("Yield %") for both elevated ionic strength formulations TF1 (95±7.4%) and TF2 (93±7.4%) are significantly higher than the recovery using the control formulation CF (77±6.6%).

TABLE 2

AAV VECTOR RECOVERY AT PROCESS SCALE

Experiment	Formulation	μ (mM)	Target (vg/mL)	Actual (vg/mL)	Yield % (RSD)
1	CF	160	2.5E13	1.93E13	77 (6.6)
1	TF1	310	2.5E13	2.38E13	95 (7.4)
1	TF2	510	2.5E13	2.33E13	93 (7.4)
2	CF	160	6.7E13	3.98E13	59 (6.0)
2	TF2	510	6.7E13	6.42E13	96 (4.4)
3	CF (−Bz)	160	3.6E13	2.46E13	68 (11)
3	CF (+Bz)	160	3.6E13	3.29E13	91 (12)

In Experiment 2, AAV2-AADC is concentrated to a higher target value (6.7×10^{13} vg/mL) in CF or TF2. Vector recovery using TF2 (96±4.4%) is again significantly higher than recovery using CF (59±6.0%). Within the variability of the assays used, vector was recovered fully at both target concentrations using TF2, indicating that aggregation was prevented. In contrast, significant aggregation was observed at both target concentrations using CF, and the extent of aggregation (i.e. loss following 0.22 μm filtration) was higher at the higher target vector concentration. In an additional experiment (not shown), 50 μL samples of AAV2 vector are taken following concentration but prior to the 0.22 μm filtration step of Experiment 2, and examined by light microscopy. Vector concentrated in CF contains obvious amounts of visible material (not shown), while no such material is seen in vector concentrated in TF2.

Experiment 3 examines the effect of prior nuclease digestion of purified vector on aggregation. In the absence of nuclease digestion recovery of AAV2-AADC in CF is 68±11%, similar to the recoveries in Experiments 1 and 2. In contrast, purified vector treated with nuclease and then concentrated in CF gives higher recovery (91±12%). These prep scale results reflect the same effect of nuclease digestion shown in FIG. **2** using the "dilution-stress" (analytical scale) method.

The results presented in Table 2 demonstrate that the methods and compositions of the present invention increase the

recovery of AAV vector recovery. For example, in various embodiments of the present invention, recovery is improved from less than about 80% to at least about 85%, 90%, 95% or more.

AAV Stability and Activity Following Storage or Freeze-Thaw Cycling

Croyle and coworkers reported a significant loss of titer of AAV and adenovirus following multiple freeze-thaw cycling in sodium phosphate buffer, and demonstrated that the better pH buffering provided by potassium phosphate during freeze-thaw cycling prevented titer loss. Croyle, M. A. et al. (2001) *Gene Therapy* 8: 1281-1290. Results of our freeze-thaw stability study using sodium phosphate support these findings. We find that while 150 mM sodium phosphate provides sufficient ionic strength to prevent aggregation during preparation and non-frozen storage of concentrated AAV2-AADC vector, even a single freeze-thaw cycle at −20 or −80° C. results in aggregation.

AAV stability after storage or freeze-thaw (F/T) cycling is assessed in buffers of the present invention as follows. The concentrated vectors prepared in CF, TF1, and TF2 (Table 2, Experiment 1) are subjected to a short stability study to investigate whether aggregation will occur during refrigerated storage, or following multiple freeze-thaw (F/T) cycles. Aggregation is assessed by DLS using undiluted samples, and Rh values >20 nm are deemed to indicate the occurrence of some level of aggregation.

TABLE 3

STABILITY OF AAV2 VECTORS

	Particle radius - Rh (nm)							
Formula-		4° C.	−20° C.			−80° C.		
tion	Pre	5 d	1 F/T	5 F/T	10 F/T	1 F/T	5 F/T	10 F/T
CF	14.5	27.0	22.4	56.1	94.5	20.6	57.5	141
TF1	13.8	16.3	TH	TH	TH	TH	TH	TH
TF2	13.8	14.4	14.2	14.0	14.1	13.8	21.3	50.9

Pre: DLS radius measured immediately following 0.2 μm filtration.
Vector concentrations (vg/mL): CF: 1.93E13, TF1: 2.38E13, TF2: 2.33E13.
TH: signal intensity is too high to measure because of extensive aggregation.

As shown in Table 3, AAV2-AADC vector prepared in CF shows some aggregation after 5 days of storage at 4° C., as well as following one or more F/T cycles at −20 or −80° C. For vector prepared in TF1, no aggregation occurs after 5 days at 4° C., but aggregation occurs following a single F/T cycle at −20 or −80 ° C. as indicated by a DLS signal intensity that is too high to measure. Visual inspection of these samples reveals slight cloudiness, which is consistent with aggregation. For vector prepared in TF2, no aggregation is observed at 4° C., or following up to 10 F/T cycles at −20° C. Some aggregation is observed following 5 and 10 F/T cycles at −80° C.

AAV activity after storage or F/T cycling in TF2 is assessed as follows. As described above, the high ionic strength, isotonic formulation TF2 effectively prevents vector aggregation during concentration and storage, and therefore represents a promising candidate for further study. An important question is whether preparation and storage of the vector in high ionic strength TF2 would adversely affect its functional activity. To assess this, assays are performed to measure the infectious titer and the transduction efficiency of vectors prepared and stored for an extended period of time in TF2.

For infectivity, a highly sensitive infectivity assay capable of detecting single infectious events is used. Zhen, Z. et al. (2004) *Human Gene Ther.* 15: 709-715. AAV2-AADC is prepared in TF2 at a concentration of 6.4×10^{13} vg/mL. After being stored for 45 days at 4° C. the preparation has a vector genome to infectious unit ratio (vg/IU) of 13, compared to a value of 16 vg/IU for the reference vector. This difference is not significant given the reported variability of this assay (RSD ~50%).

Transduction efficiency is assessed by measuring the expression of AADC protein by ELISA following transduction of D7/4 cells. FIG. **3** shows no significant difference between vector prepared in TF2 and the reference control for vector input ranging from 10 to 10^5 vg/cell. Together, these data indicate that preparation and storage of AAV2 vectors in high ionic strength TF2 does not have a deleterious effect on vector infectivity or transduction efficiency.

Conclusion

The effect of ionic strength (μ) on virus particle interactions is determined to elucidate the mechanism of vector aggregation. The ionic strength of neutral-buffered isotonic saline (μ=150 mM) is insufficient to prevent aggregation of AAV2 vectors purified by gradient ultracentrifugation or by cation exchange chromatography at concentrations exceeding ~10^{13} particles/mL. Inclusion of sugars (sorbitol, sucrose, mannitol, trehalose, glycerol) at concentrations up to 5% (w/v) or of surfactants Tween80® (1%) or Pluronic® F68 (10%) does not prevent aggregation of vector particles.

In contrast, vector particles remain soluble when elevated ionic strength solutions (μ>200 mM) are used during purification and for final vector formulation. Elevated ionic strength solutions using isotonic excipient concentrations for in vivo administration are prepared with salts of multivalent ions, including sodium citrate, sodium phosphate, and magnesium sulfate. An isotonic formulation containing 10 mM Tris, 100 mM sodium citrate, 0.001 % Pluronic® F68, pH 8.0 (μ~500 mM) enables concentration of AAV2-AADC vectors to 6.4×10^{13} vg/mL with no aggregation observed during preparation and following ten freeze-thaw cycles at −20° C. See Table 3, below, and accompanying discussion. AAV2-AADC vectors prepared and stored for an extended period in elevated ionic strength formulation retain high infectivity titer (13 IU/vg) and transduction efficiency.

Nuclease treatment of purified AAV2 vectors reduces the degree of vector aggregation, implicating vector surface nucleic acid impurities in inter-particle interactions. Hence, purification methods to efficiently remove vector surface residual nucleic acids, coupled with the use of elevated ionic strength isotonic formulations, are useful methods to prevent AAV2 vector aggregation.

EXAMPLE 1

AAV Purification Methods

AAV2 vectors expressing human coagulation factor IX (FIX) or human amino acid decarboxylase (AADC) are produced by triple transfection of HEK293 cells as previously described (Matsushita, T. et al. (1998) *Gene Therapy* 5: 938-945), with modifications. For the large scale preparations, cells are cultured and transfected in 850 mm^2 roller bottles (Corning). Vectors are purified by one of three methods.

In purification Method 1, modified from Matsushita, transfected HEK293 cells in roller bottles are collected by centrifugation (1000 g, 15 min), resuspended in 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2, and lysed by three freeze/thaw cycles (alternating an ethanol/dry ice bath and a 37° C. water bath). The cell lysate is clarified by cen-

trifugation (8,000 g, 15 min). The supernatant is then diluted to 200 mM NaCl by addition of 10 mM sodium phosphate, pH 7.2, and digested with Benzonase® (Merck, Purity Grade 1; 200 U/mL, 1 h, 37° C.). The lysate is adjusted to 25 mM $CaCl_2$ using a 1M stock solution, and incubated at 4° C. for one hour.

The mixture is centrifuged (8,000 g, 15 min), and the supernatant containing vector is collected. To precipitate virus from the clarified cell lysate, polyethylene glycol (PEG8000) is added to a final concentration of 8%, the mixture incubated at 4° C. for three hours, and then centrifuged (8,000 g, 15 min). The pellets containing vector are re-suspended with mixing in 0.15M NaCl, 50 mM Hepes, 25 mM EDTA, pH 8.0 and incubated at 4° C. for 16 hours. The resuspended material is pooled, and solid cesium chloride is added to a final density of 1.40 gm/ml. Vector is then banded by ultracentrifugation (SW28, 27,000 rpm, 24 h, 20° C.) using a Beckman model LE-80 centrifuge. The centrifugation tubes are fractionated, and densities from 1.38 to 1.42 gm/mL containing vector are pooled. This material is banded a second time by ultracentrifugation (NVT65 rotor, 65,000 rpm, 16 h, 20° C.), and fractions containing purified AAV2 vectors are pooled. To concentrate vector and to perform buffer exchange, vectors in concentrated cesium chloride solution are subjected to ultrafiltration/diafiltration (UF/DF) by tangential flow filtration as described below (Example 2).

In purification Method 2, cell harvests containing AAV are microfluidized and filtered sequentially through 0.65 and 0.22 μm filters (Sartorius). Virus is purified from the clarified cell lysates by cation exchange chromatography using Poros HS50 resin as previously described. U.S. Pat. No. 6,593,123. For the nuclease digestion described in FIG. **2**, column-purified vectors are incubated (4 h, RT) with 100 U/mL Benzonase and 10 U/mL DNAse I (RNAse free, Roche Diagnostics, Indianapolis, Ind.).

For purification Method 3, AAV2 vectors purified by cation exchange chromatography are subjected to an additional cesium chloride gradient ultracentrifugation step (SW28, 27,000 rpm, 20 h) to remove empty capsids prior to UF/DF.

Real time quantitative PCR (Q-PCR) is used to quantify AAV preparations as previously described. Sommer, J. M. et al. (2003) *Mol. Therapy* 7: 122-128. Vectors purified by each of the three methods are analyzed by SDS-PAGE/silver staining analysis, and in all cases VP1, VP2 and VP3 are present in the expected ratios, with the capsid proteins representing >95% of total proteins as determined by scanning densitometry. However, unlike gradient-purified AAV2 vectors purified using Methods 1 and 3, vectors purified by Method 2 (column chromatography) contain empty capsids, ranging from 3-10 empty capsids per vector genome.

EXAMPLE 2

Ultrafiltration and Diafiltration to Detect AAV Aggregation

Disposable hollow fiber tangential flow filtration devices (Amersham BioSciences 8" Midgee, 100 kDa nominal pore size) are used to concentrate and diafilter AAV2 vectors purified by the methods described above, and for the UF/DF experiments described in Table 2. For all UF/DF procedures a volume of diafiltration buffer corresponding to 10× the product volume is used, and it is added in ~1 mL increments to approximate continuous diafiltration. Using this method, the calculated residual CsCl after diafiltration is <0.5 mM.

The following three formulations were used for UF/DF: Control Formulation (CF: 140 mM sodium chloride, 10 mM sodium phosphate, 5% sorbitol, pH 7.3); Test Formulation 1 (TF1: 150 mM sodium phosphate, pH7.5); and Test Formulation 2 (TF2: 100 mM sodium citrate, 10 mM Tris, pH8.0). For Experiment 1 shown in Table 2, diafiltration is performed at a volume corresponding to a vector concentration of 1×10^{13} vg/mL, and following diafiltration the volume is reduced to a value corresponding to 2.5×10^{13} vg/mL (assuming no vector loss).

For Experiment 2, diafiltration is performed at a volume corresponding to a 2×10^{13} vg/mL, and the volume is then reduced to a value corresponding to 6.7×10^{13} vg/mL.

For Experiment 3 (CF±Bz), AAV2-AADC (approximately 1.2×10^{14} vg) is first diafiltered into TF1 (a formulation compatible with nuclease activity) and then passed through a 0.22 μm filter. The titer of this material is determined, and the volume is adjusted to correspond to a concentration of 1×10^{13} vg/mL. To 10 mL of this material, $MgCl_2$ is added to a concentration of 2 mM, and then divided into two equal aliquots. One aliquot is incubated with Benzonase (200 U/mL, 4 h, RT), and the second is mock-incubated. Each aliquot is then diafiltered at a volume corresponding to a vector concentration 2×10^{13} vg/mL, and then concentrated to a 3.6×10^{13} vg/mL target. Following all UF/DF protocols, Pluronic® F-68 (BASF Corp., Mount Olive, N.J.) from a 1% stock is added to the vector product to a final concentration of 0.001%, and the solution is passed through a 0.22 μm syringe filter (Sartorius). All UF/DF procedures are performed in a laminar flow cabinet.

EXAMPLE 3

Measurement of Vector Aggregation by Dynamic Light Scattering

Purified vectors are analyzed for aggregation by dynamic light scattering (DLS) using a Protein Solutions DynaPro 99 (λ=825.4 nm). Primary data (particle radius—Rh, average value measured over 30 cycles, 10 cycles/min) are used for all analyses reported. A "dilution-stress" method is used to assess the effect of varying excipients on vector aggregation. In this method, 80 μL of test diluent is added to 20 μL of vector solution with mixing in the actual cuvette used for DLS measurement, and data collection is initiated within 10 seconds of mixing. Prior to addition of test diluents, the Rh value for AAV2 vector preparations is measured and confirmed to be <15 nm to ensure that the starting material is monomeric. Samples that are not 100% monomeric are passed through a 0.22 μm syringe disc filter (Sartorius, low protein binding) to remove aggregates.

The osmolarity and ionic strength values given in FIGS. **1** and **2** are calculated using all excipients present in the mixture (i.e. weighted: test diluent (80%) and starting vector formulation (20%)). Osmolarity is calculated according to the equation: Osmolarity=Σc_i, where c_i is the molar concentration of each solute species. The ionic strength (μ) is calculated according to the equation: $\mu=\frac{1}{2}\Sigma c_i z_i^2$, where z_i is the charge on each species. In conditions that resulted in vector aggregation (e.g. low μ) a progressive increase in Rh is observed over the course of data collection. To validate the use of the average Rh measured over the 3 minute interval following dilution as a reliable measure of aggregation, the average rate of increase of Rh (ΔRh/Δt) over the same time interval is also

determined (not shown). Analysis of ΔRh/Δt gives results concordant with those obtained using the average Rh value reported in FIGS. **1** and **2**.

EXAMPLE 4

AAV Virion Infectivity

Infectivity of AAV2-AADC vectors is determined using a highly sensitive assay as previously described. Zhen, Z. et al. (2004) *Human Gene Ther.* 15: 709-715. Briefly, samples are serially diluted (10-fold dilutions, 10 replicates/dilution) and added to D7/4 cells (modified HeLa cells expressing AAV rep and cap) grown in 96 well tissue culture plates (Falcon, cat. #353227) in DMEM medium containing 10% FBS. Adenovirus (Ad-5, 100 vp/cell) is added to each well to provide helper functions. After 48 h, replication of AAV vector in each well is quantified by Q-PCR using transgene-specific primers and probes, and the frequency of infection at limiting dilution is analyzed by the Karber method to calculate the infectivity titer. The test sample is run concurrently with an AAV2-AADC reference previously prepared in CF and stored at −80° C.

The transduction efficiency of AAV2 vectors is quantified by a whole cell ELISA. D7/4 cells grown in 96 well plates are infected with 10-fold serial dilutions of the test sample and reference vector, corresponding to 10 to 10^5 vg/cell input (5 replicates/dilution). After 48 h, the culture medium is removed, and cells are washed twice with 200 μL PBS (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.2). Cells are then permeabilized and fixed by addition of 100 μL of PBS containing 0.5% Triton X-100 and 4% paraformaldehyde to each well (15 min). The fixing solution is removed, and the cells are washed twice with PBS containing 0.5% Triton X-100. Non-specific sites are blocked by adding PBS containing 3% bovine serum albumin (BSA) and 0.5% Triton X-100 (60min).

After washing, cells are incubated for one hour with rabbit anti-AADC IgG antibody (Chemicon, AB136), and washed. Cells are then incubated for one hour with alkaline phosphatase-conjugated goat anti-rabbit IgG, and washed. Antibodies are diluted 1:1000 in PBS containing 1% BSA, 0.5% Triton X-100. Substrate (PNPP, Pierce, cat. #34047) is then added (1 mg/mL in 1×diethanolamine substrate buffer, Pierce, cat. #34064), and after incubation for 30 min the concentration of cleaved substrate is measured spectrophotometrically (λ=405 nm). Human AADC expression as a function of vector input is fitted using a spline curve (SigmaPlot). The AAV2-AADC reference vector is measured concurrently with the test sample.

While preferred illustrative embodiments of the present invention are described, it will be apparent to one skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is intended in the appended claims to cover all such changes and modifications that fall within the true spirit and scope of the invention.

All publications, patents and patent applications referred to herein are hereby incorporated by reference in their entireties.

We claim:

1. A method of preventing aggregation of recombinant adeno-associated virus (rAAV) virions in a purified preparation of rAAV virions, comprising:

1) providing a lysate comprising rAAV virions;

2) purifying rAAV virions from the lysate using ultracentrifugation and/or chromatography, wherein said virions are purified; and

3) adding one or more salts of multivalent ions selected from the group consisting of citrate, phosphate, sulfate and magnesium to said purified virions to produce a preparation of virions with an ionic strength of at least 200 mM, wherein the concentration of purified rAAV virions in said preparation exceeds 1×10^{13} vg/ml up to 6.4×10^{13} vg/ml; and wherein the pH of the purified preparation of rAAV virions is between 7.5 and 8.0.

2. The method of claim **1**, further comprising treating said purified virions with a nuclease.

3. The method of claim **2**, wherein the nuclease is an endonuclease from *Serratia marcescens*.

4. The method of claim **1**, wherein the multivalent ion is citrate.

5. The method of claim **1**, wherein the osmolarity of the preparation of virions after addition of the one or more salts of multivalent ions is no greater than about 280 mOsm.

6. The method of claim **1**, wherein, after addition of the one or more salts of multivalent ions, the average particle radius (Rh) of the virions in the preparation of virions is less than about 20 nm as measured by dynamic light scattering.

7. The method of claim **1**, wherein, after addition of the one or more salts of multivalent ions, recovery of the virions is at least about 90% following filtration of the preparation of virions through a 0.22 μm filter.

8. The method of claim **2**, wherein rAAV virions are purified from the lysate using cesium chloride gradient ultracentrifugation.

9. The method of claim **2**, wherein rAAV virions are purified from the lysate using cation exchange chromatography.

10. The method of claim **2**, wherein rAAV virions are purified from the lysate using cation exchange chromatography and cesium chloride gradient ultracentrifugation.

11. The method of claim **2**, further comprising diafiltering the purified rAAV virions to achieve an ionic strength of at least 200 mM.

* * * * *

Exhibit C

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use ELEVIDYS® safely and effectively. See full prescribing information for ELEVIDYS.

ELEVIDYS (delandistrogene moxeparvovec-rokl) suspension, for intravenous infusion
Initial U.S. Approval: 2023

----------RECENT MAJOR CHANGES----------

Indication and Usage (1)	6/2024
Dosage and Administration (2)	8/2024
Warnings and Precautions (5)	6/2024

----------INDICATIONS AND USAGE----------

ELEVIDYS is an adeno-associated virus vector-based gene therapy indicated in individuals at least 4 years of age:

- For the treatment of Duchenne muscular dystrophy (DMD) in patients who are ambulatory and have a confirmed mutation in the *DMD* gene. (1,12.2,14)
- For the treatment of DMD in patients who are non-ambulatory and have a confirmed mutation in the *DMD* gene (1,12.2)
  The DMD indication in non-ambulatory patients is approved under accelerated approval based on expression of ELEVIDYS micro-dystrophin (noted hereafter as "micro-dystrophin"). Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial(s). (1, 12.2)

----------DOSAGE AND ADMINISTRATION----------

ELEVIDYS is for single-dose intravenous infusion only.

- Select patients for treatment with ELEVIDYS with anti-AAVrh74 total binding antibody titers <1:400. (2.1)
- Recommended dosage: 10 to 70 kg: 1.33×10^{14} vector genomes (vg) per kg of body weight; 70 kg or greater: 9.31×10^{15} vg. (2.2)
- There is limited safety data available in non-ambulatory patients weighing 70 kg or greater, who received the maximum dose of ELEVIDYS, 9.31×10^{15} vg, in clinical trials. (2.2)
- Postpone in patients with concurrent infections until the infection has resolved. (2.2)
- Assess liver function, platelet counts and troponin-I before ELEVIDYS infusion. (2)
- One day prior to infusion, initiate a corticosteroid regimen for a minimum of 60 days. Recommend modifying corticosteroid dose for patients with liver function abnormalities. (2.2)
- Administer as an intravenous infusion over 1-2 hours. Infuse at a rate of less than 10 mL/kg/hour. (2.4)

----------DOSAGE FORMS AND STRENGTHS----------

- ELEVIDYS is a suspension for intravenous infusion with a nominal concentration of 1.33×10^{13} vg/mL. (3)
- ELEVIDYS is provided in a customized kit containing ten to seventy 10 mL single-dose vials, with each kit constituting a dosage unit based on the patient's body weight. (3)

----------CONTRAINDICATIONS----------

- ELEVIDYS is contraindicated in patients with any deletion in exon 8 and/or exon 9 in the *DMD* gene. (4)

----------WARNINGS AND PRECAUTIONS----------

- Infusion-related Reactions: Infusion-related reactions, including hypersensitivity reactions and anaphylaxis, have occurred. Monitor during administration and for at least 3 hours after end of infusion. If symptoms occur, slow or stop the infusion and give appropriate treatment. Once symptoms resolve, restart infusion at a slower infusion rate. Discontinue infusion for anaphylaxis. (2.4, 5.1)
- Acute Serious Liver Injury: Acute serious liver injury has been observed. Monitor liver function before ELEVIDYS infusion, and weekly for the first 3 months after ELEVIDYS infusion. Continue monitoring until results are unremarkable. If acute serious liver injury is suspected, a consultation with a specialist is recommended. (5.2)
- Immune-mediated Myositis: Patients with deletions in the *DMD* gene in exons 1 to 17 and /or exons 59 to 71 may be at risk for severe immune-mediated myositis reaction. Consider additional immunomodulatory treatment (immunosuppressants [e.g., calcineurin-inhibitor] in addition to corticosteroids) if symptoms of myositis occur (e.g., unexplained increased muscle pain, tenderness, or weakness). (5.3)
- Myocarditis: Myocarditis and troponin-I elevations have been observed. Monitor troponin-I before ELEVIDYS infusion, and weekly for the first month after ELEVIDYS infusion. (5.4)
- Pre-existing Immunity against AAVrh74: Perform baseline testing for presence of anti-AAVrh74 total binding antibodies prior to ELEVIDYS administration. (5.5)

----------ADVERSE REACTIONS----------

Most common adverse reactions across studies (incidence ≥5%) were vomiting and nausea, liver injury, pyrexia, and thrombocytopenia. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Sarepta Therapeutics, Inc., at 1-888-SAREPTA (1-888-727-3782) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See 17 for PATIENT COUNSELING INFORMATION

Revised: 8/2024

FULL PRESCRIBING INFORMATION: CONTENTS*

* Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

ELEVIDYS is indicated for the treatment of Duchenne muscular dystrophy (DMD) in individuals at least 4 years of age:

- For patients who are ambulatory and have a confirmed mutation in the *DMD* gene *[see Clinical Pharmacology (12.2), Clinical Studies (14)*]
- For patients who are non-ambulatory and have a confirmed mutation in the *DMD* gene.

The DMD indication in non-ambulatory patients is approved under accelerated approval based on expression of ELEVIDYS micro-dystrophin (noted hereafter as "micro-dystrophin") in skeletal muscle. Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial(s). *[see Clinical Pharmacology (12.2)].*

2 DOSAGE AND ADMINISTRATION

For single-dose intravenous infusion only.

2.1 Patient Selection

Select patients for treatment with ELEVIDYS with anti-AAVrh74 total binding antibody titers <1:400. An FDA-authorized test for the detection of anti-AAVrh74 total binding antibodies is not currently available. Currently available tests may vary in accuracy and design.

2.2 Dose

The recommended dose of ELEVIDYS is 1.33×10^{14} vector genomes per kilogram (vg/kg) of body weight (or 10 mL/kg body weight) for patients weighing less than 70 kg or 9.31×10^{15} vg total fixed dose for patients weighing 70 kg or greater. There is limited safety data available in non-ambulatory patients weighing 70 kg or greater, who received the maximum dose of ELEVIDYS, 9.31×10^{15} vg, in clinical trials.

For the number of vials required, refer to Table 10 *[see How Supplied/Storage and Handling (16.1)]*.

Calculate the dose as follows:

ELEVIDYS dose (in mL) = patient body weight (rounded to the nearest kilogram) x 10

The multiplication factor 10 represents the per kilogram dose (1.33×10^{14} vg/kg) divided by the amount of vector genome copies per mL of the ELEVIDYS suspension (1.33×10^{13} vg/mL).

Number of ELEVIDYS vials needed = ELEVIDYS dose (in mL) divided by 10.

Example: Calculation of volume needed for a 19.5 kg patient
19.5 kg rounded to the nearest kilogram = 20 kg
20 kg × 10 = 200 mL

Number of ELEVIDYS vials needed = 200 divided by 10, rounded to the nearest number of vials = 20 vials

Prior to ELEVIDYS infusion:
Due to the increased risk of serious systemic immune response, postpone ELEVIDYS in patients with infections until the infection has resolved. Clinical signs or symptoms of infection should not be evident at the time of ELEVIDYS administration *[see Patient Counseling Information (17)]*.

Assess liver function *[see Dosage and Administration (2.4), Warnings and Precautions (5.2), Use in Specific Populations (8.6)]*.

Obtain platelet count and troponin-I levels *[see Dosage and Administration (2.4), Warnings and Precautions (5.4)]*.

Measure baseline anti-AAVrh74 antibody titers using a Total Binding Antibody enzyme-linked immunosorbent assay (ELISA) *[see Dosage and Administration (2), Clinical Pharmacology (12.6)]*.

ELEVIDYS administration is not recommended in patients with elevated anti-AAVrh74 total binding antibody titers (≥1:400). Re-administration of ELEVIDYS is not recommended *[see Warnings and Precautions (5.5), Clinical Pharmacology (12.6)]*.

Immune responses to the AAVrh74 vector can occur after administration of ELEVIDYS *[see Clinical Pharmacology (12.6)]*. To reduce the risk associated with an immune response, corticosteroids should be administered starting 1 day prior to ELEVIDYS infusion. Initiate a corticosteroid regimen following the appropriate schedule (see Table 1). This regimen is recommended for a minimum of 60 days after the infusion, unless earlier tapering is clinically indicated. Table 2 includes the recommended corticosteroid regimen dose modification for patients with liver function abnormalities following ELEVIDYS infusion. If acute serious liver injury is suspected, a consultation with a specialist is recommended.

For patients previously taking corticosteroids at baseline, taper off the additional peri-ELEVIDYS corticosteroids (back to baseline corticosteroid dose) over 2 weeks, or longer as needed. For patients not previously taking corticosteroids at baseline, taper the added peri-ELEVIDYS corticosteroids off (back to no corticosteroids) over 4 weeks, or longer, as needed, and the corticosteroids should not be stopped abruptly.

Table 1: Recommended pre- and post-infusion corticosteroid dosing

Baseline corticosteroid dosing[a]	Peri-ELEVIDYS infusion corticosteroid dose (prednisone equivalent) [b]	Recommended maximum total daily dose (prednisone equivalent)[b]
Daily or intermittent dose	Start 1 day prior to infusion: 1 mg/kg/day (and continue baseline dose)	60 mg/day
High dose for 2 days per week	Start 1 day prior to infusion: 1 mg/kg/day taken on days without high-dose corticosteroid treatment (and continue baseline dose)	60 mg/day
Not on corticosteroids	Start 1 week prior to infusion: 1.5 mg/kg/day	60 mg/day

[a] Patient continues to receive this dose
[b] Corticosteroids other than prednisone and prednisolone have not been studied for use as a peri-ELEVIDYS infusion corticosteroid

Table 2: Recommended corticosteroid regimen dose modification for liver function abnormalities following ELEVIDYS infusion[a]

Peri-ELEVIDYS infusion corticosteroid dosing	Modified corticosteroid dose following ELEVIDYS infusion (prednisone equivalent)[b]	Recommended maximum total daily dose (prednisone equivalent)[b]
Baseline + 1 mg/kg/day	Increase to 2 mg/kg/day (and continue baseline dose)	120 mg/day
Baseline + 1 mg/kg/day taken on days without high-dose corticosteroid treatment	Increase to 2 mg/kg/day taken on days without high-dose corticosteroid treatment (and continue baseline dose)	120 mg/day
1.5 mg/kg/day	Increase from 1.5 mg/kg/day to 2.5 mg/kg/day	120 mg/day

[a] GGT >= 150 U/L and/or other clinically significant liver function abnormalities (e.g., total bilirubin > 2 x ULN) following infusion. For GGT or bilirubin elevations that do not respond to these oral corticosteroid increases, IV bolus corticosteroids may be considered.
[b] Corticosteroids other than prednisone and prednisolone have not been studied for use as a peri-ELEVIDYS infusion corticosteroid.

2.3 Preparation

General precautions

- Prepare ELEVIDYS using aseptic technique.
- Verify the required dose of ELEVIDYS based on the patient's body weight.
- Confirm that the kit contains sufficient number of vials to prepare the ELEVIDYS infusion for the patient.
- Visually inspect parenteral drug products for particulate matter and discoloration prior to administration, whenever suspension and container permit. ELEVIDYS may contain white to off-white particles.

Recommended supplies and materials:

- 60 mL siliconized polypropylene syringes
- 21-gauge or smaller stainless steel needles

Preparing ELEVIDYS infusion

1. Thaw ELEVIDYS before use.
    - When thawed in the refrigerator, ELEVIDYS vials are stable for up to 14 days in the refrigerator (2°C to 8°C [36º F to 46º F]) when stored in the upright position.
    - Frozen ELEVIDYS vials will thaw in approximately 2 hours when placed at room temperature (up to 25°C [77ºF]) when removed from original packaging.
    - Thawed ELEVIDYS in vials or syringes is stable for up to 24 hours at room temperature (up to 25°C [77ºF]).
2. Inspect vials to ensure no ice crystals are present prior to preparation.
3. When thawed, swirl gently.
    - Do not shake.
    - Do not refreeze.
    - Do not place back in the refrigerator.
4. Visually inspect each vial of ELEVIDYS. ELEVIDYS is a clear, colorless liquid that may have some opalescence. ELEVIDYS may contain white to off-white particles.
    - Do not use if the suspension in the vials is cloudy or discolored.

5. Remove the plastic flip-off cap from the vials and disinfect the rubber stopper with a sterilizing agent (e.g., alcohol wipes).
6. Withdraw 10 mL of ELEVIDYS from each vial provided in the customized ELEVIDYS kit (refer to Table 10).
    - Do not use filter needles during preparation of ELEVIDYS.
    - Multiple syringes will be required to withdraw the required volume.
    - Remove air from the syringes and cap the syringes.
7. Maintain syringes at room temperature prior to and during administration.

2.4 Administration

Recommended supplies and materials:

- Syringe infusion pump
- 0.2-micron PES* in-line filter with a large surface area. To avoid the risk of occlusions, the use of smaller pediatric in-line filters (e.g., less than 10 cm^2 surface area) is not recommended.
- PVC* (non-DEHP*) IV infusion tubing, and polyurethane catheter

*PVC = Polyvinyl chloride, DEHP = Di(2-ethylhexyl) phthalate, PES = Polyether sulfone

Administer ELEVIDYS as a single-dose intravenous infusion through a peripheral venous catheter:

ELEVIDYS should be administered in a setting where treatment for infusion-related reactions is immediately available *[see Warnings and Precautions (5.1)]*. Do not infuse ELEVIDYS at a rate of 10 mL/kg/hour or faster.

Consider application of a topical anesthetic to the infusion site prior to administration of IV insertion.

Recommend inserting a back-up catheter.

1. Flush the intravenous access line with 0.9% Sodium Chloride Injection prior to the ELEVIDYS infusion at the same infusion rate.
2. Administer ELEVIDYS via intravenous infusion using a syringe infusion pump with an in-line 0.2-micron filter at a duration of approximately 1 to 2 hours, or longer at care team discretion, through a peripheral limb vein.
3. Infuse at a rate of less than 10 mL/kg/hour.
    - Do not administer ELEVIDYS as an intravenous push.
    - Do not infuse ELEVIDYS in the same intravenous access line with any other product.
    - Use ELEVIDYS within 12 hours after drawing into syringe. Discard the ELEVIDYS-containing syringe(s) if infusion of the drug has not been completed within the 12-hour timeframe.
4. In the event of an infusion-related reaction during administration *[see Warnings and Precautions (5.1)]:*

- Slow or stop the infusion based on patient's clinical presentation.
- Discontinue infusion for anaphylaxis.
- Administer treatment as needed to manage infusion-related reaction.
- ELEVIDYS infusion may be restarted at a lower rate after the infusion-related reaction has resolved at the discretion of the physician, based on severity of patient's clinical presentation.
- If the ELEVIDYS infusion needs to be stopped and restarted, ELEVIDYS should be infused within 12 hours after drawing into the syringe *[see How Supplied/Storage and Handling (16.2)]*.

5. Flush the intravenous access line with 0.9% Sodium Chloride Injection after the ELEVIDYS infusion.
    - Discard unused ELEVIDYS *[see How Supplied/Storage and Handling (16.2)]*.
    - Dispose of the needle and syringe *[see How Supplied/Storage and Handling (16.2)]*.

Monitoring Post-ELEVIDYS Administration

- Assess liver function (clinical exam, GGT, and total bilirubin) weekly for the first 3 months. Continue monitoring if clinically indicated, until results are unremarkable (normal clinical exam, GGT and total bilirubin levels return to near baseline levels) *[see Warnings and Precautions (5.2), Specific Populations (8.6)]*.
- Obtain platelet counts weekly for the first two weeks *[see Adverse Reactions (6.1)]*. Continue monitoring if clinically indicated.
- Measure troponin-I weekly for the first month *[see Warnings and Precautions (5.4)]*. Continue monitoring if clinically indicated.

3 DOSAGE FORMS AND STRENGTHS

ELEVIDYS is a preservative-free, sterile, clear, colorless liquid that may have some opalescence and may contain white to off-white particles.

ELEVIDYS is a suspension for intravenous infusion with a nominal concentration of 1.33×10^{13} vg/mL.

ELEVIDYS is provided in a customized kit containing ten to seventy 10 mL single-dose vials, with each kit constituting a dosage unit based on the patient's body weight *[see How Supplied/Storage and Handling (16.1)]*.

The intravenous dosage is determined by patient body weight, with a recommended dose of 1.33×10^{14} vector genomes (vg)/kg for patients weighing 10 to 70 kg, and a maximum of 9.31×10^{15} vg for patients 70 kg or greater.

4 CONTRAINDICATIONS

ELEVIDYS is contraindicated in patients with any deletion in exon 8 and/or exon 9 in the *DMD* gene *[see Warnings and Precautions (5.3)]*.

5 WARNINGS AND PRECAUTIONS

5.1 Infusion-related Reactions

Infusion-related reactions, including hypersensitivity reactions and anaphylaxis, have occurred during or up to several hours following ELEVIDYS administration. Closely monitor patients during and for at least 3 hours after the end of infusion for signs and symptoms of infusion-related reactions including tachycardia, tachypnea, lip swelling, difficulty breathing, nasal flaring, urticaria, flushing, lip pruritus, rash, cheilitis, vomiting, nausea, rigors and pyrexia.

ELEVIDYS should be administered in a setting where treatment for infusion-related reactions is immediately available.

In the event of an infusion-related reaction, administration of ELEVIDYS may be slowed or stopped based on the severity of the patient's clinical presentation. Administer treatment as needed to manage infusion-related reactions based on the severity of patient's signs and symptoms. *[see Dosage and Administration (2.4)]*. If the infusion was stopped, ELEVIDYS infusion may be restarted at a lower rate once patient's symptoms have resolved, at the discretion of the physician. Discontinue infusion for anaphylaxis.

5.2 Acute Serious Liver Injury

Acute serious liver injury has been observed with ELEVIDYS. Administration of ELEVIDYS may result in elevations of liver enzymes (e.g., GGT, ALT) and total bilirubin, typically seen within 8 weeks.

Patients with preexisting liver impairment, chronic hepatic condition or acute liver disease (e.g., acute hepatic viral infection) may be at higher risk of acute serious liver injury. Postpone ELEVIDYS administration in patients with acute liver disease until resolved or controlled. Patients with hepatic impairment, acute liver disease, chronic hepatic condition or elevated GGT have not been studied in clinical trials with ELEVIDYS *[see Specific Populations (8.6)]*.

In clinical studies, liver function test increased (including increases in GGT, GLDH, ALT, AST, or total bilirubin) was commonly reported typically within 8 weeks following ELEVIDYS infusion, with the majority of cases being asymptomatic *[see Adverse Reactions (6.1)]*. Cases resolved spontaneously or with systemic corticosteroids and resolved without clinical sequelae within 2 months. No cases of liver failure were reported.

Prior to ELEVIDYS administration, perform liver enzyme test *[see Dosage and Administration (2.2)]*. Monitor liver function (clinical exam, GGT, and total bilirubin) weekly for the first 3 months following ELEVIDYS infusion. Continue monitoring if clinically indicated, until results are unremarkable (normal clinical exam, GGT and total bilirubin levels return to near baseline levels) *[see Dosage and Administration (2.4)]*.

Systemic corticosteroid treatment is recommended for patients before and after ELEVIDYS infusion *[see Dosage and Administration (2.2)]*. Adjust corticosteroid regimen when indicated *[see Dosage and Administration (2.2)]*. If acute serious liver injury is suspected, a consultation with a specialist is recommended.

5.3 Immune-mediated Myositis

In clinical trials, immune-mediated myositis has been observed approximately 1 month following ELEVIDYS infusion in patients with deletion mutations involving exon 8 and/or exon 9 in the *DMD* gene. Symptoms of severe muscle weakness, including dysphagia, dyspnea and hypophonia, were observed. In a life-threatening case of immune-mediated myositis, symptoms resolved during hospitalization following additional immunomodulatory treatment; muscle strength gradually improved but did not return to baseline level. These immune reactions may be due to a T-cell based response from lack of self-tolerance to a specific region encoded by the transgene corresponding to exons 1-17 of the *DMD* gene.

Limited data are available for ELEVIDYS treatment in patients with mutations in the *DMD* gene in exons 1 to 17 and/or exons 59 to 71 *[see Clinical Studies (14)]*. Patients with deletions in these regions may be at risk for a severe immune-mediated myositis reaction. ELEVIDYS is contraindicated in patients with any deletion in exon 8 and/or exon 9 in the *DMD* gene due to the increased risk for a severe immune-mediated myositis reaction *[see Contraindications (4)]*.

Advise patients to contact a physician immediately if they experience any unexplained increased muscle pain, tenderness, or weakness, including dysphagia, dyspnea or hypophonia as these may be symptoms of myositis. Consider additional immunomodulatory treatment (immunosuppressants [e.g., calcineurin-inhibitor] in addition to corticosteroids) based on patient's clinical presentation and medical history if these symptoms occur.

5.4 Myocarditis

Acute serious myocarditis and troponin-I elevations have been observed following ELEVIDYS infusion in clinical trials.

If a patient experiences myocarditis, those with pre-existing left ventricle ejection fraction (LVEF) impairment may be at higher risk of adverse outcomes. Patients with moderate to severe LVEF impairment have not been studied in clinical trials with ELEVIDYS.

Monitor troponin-I before ELEVIDYS infusion and weekly for the first month following infusion *[see Dosage and Administration (2.4)]*. Continue monitoring if clinically indicated. More frequent monitoring may be warranted in the presence of cardiac symptoms, such as chest pain or shortness of breath.

Advise patients to contact a physician immediately if they experience cardiac symptoms.

5.5 Pre-existing Immunity against AAVrh74

In AAV-vector based gene therapies, preexisting anti-AAV antibodies may impede transgene expression at desired therapeutic levels. Following treatment with ELEVIDYS all patients developed anti-AAVrh74 antibodies. Perform baseline testing for the presence of anti-AAVrh74 total binding antibodies prior to ELEVIDYS administration *[see Dosage and Administration (2.1)]*.

ELEVIDYS administration is not recommended in patients with elevated anti-AAVrh74 total binding antibody titers (≥1:400).

6 ADVERSE REACTIONS

The most common adverse reactions (incidence ≥ 5%) reported in clinical studies were vomiting, nausea, liver injury, pyrexia, and thrombocytopenia.

The following clinically significant adverse reactions are described elsewhere in the labeling:

- Infusion-related reactions *[see Warnings and Precautions (5.1)]*
- Acute serious liver injury *[see Warnings and Precautions (5.2)]*
- Immune-mediated myositis *[see Warnings and Precautions (5.3)]*
- Myocarditis *[see Warnings and Precautions (5.4)]*

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The safety data described in this section reflect exposure to a one-time intravenous infusion of ELEVIDYS in 156 male patients with a confirmed mutation of the *DMD* gene in four clinical studies, including one completed open-label study, one ongoing open-label study, and two studies that included a double-blind, placebo-controlled period. Prior to ELEVIDYS infusion, patients in the ELEVIDYS treatment group had a mean age of

6.7 years (range: 3 to 20) and mean weight of 24.6 kg (range: 12.5 to 80.1). 144 patients received the recommended dose of 1.33×10^{14} vg/kg, and 12 received a lower dose. Table 3 below presents adverse reactions from these four clinical studies.

The most common adverse reactions (incidence ≥5%) across all studies are summarized in Table 3.

Adverse reactions were typically seen within the first 2 weeks (nausea, vomiting, thrombocytopenia, pyrexia), or within the first 2 months (immune-mediated myositis, liver injury). Vomiting may occur as early as on the day of the infusion.

Table 3. Adverse reactions (Incidence ≥5%) following treatment with ELEVIDYS in Clinical Studies

Adverse reactions	ELEVIDYS (N=156) %
Vomiting	65
Nausea	43
Liver injury[a]	40
Pyrexia	28
Thrombocytopenia[b c]	8

[a] Includes: AST increased, ALT increased, GGT increased, GLDH increased, GLDH level abnormal, Hepatotoxicity, Hepatic enzyme increased, Hypertransaminasemia, Liver function test increased, Liver injury, Transaminases increased, Blood bilirubin increased

[b] Includes: Thrombocytopenia, Platelet count decreased

[c] Transient, mild, asymptomatic decrease in platelet counts

In the double-blind, placebo-controlled trial, Study 3 Part 1, patients 4 to 7 years of age (N=125) received either ELEVIDYS (N=63) at the recommended dose of 1.33×10^{14} vg/kg or placebo (N=62). Table 4 below presents the most frequent adverse reactions from Study 3 Part 1.

Table 4. Adverse reactions occurring in ELEVIDYS-treated patients and at least twice more frequently than with placebo in Study 3 Part 1

Adverse reactions	ELEVIDYS (N=63) %	Placebo (N=62) %
Vomiting	64	19
Nausea	40	13
Liver injury[a]	41	8
Pyrexia	32	24
Thrombocytopenia[bc]	3	0

[a] Includes: AST increased, ALT increased, GGT increased, GLDH increased, GLDH level abnormal, Hepatotoxicity, Hepatic enzyme increased, Hypertransaminasemia, Liver function test increased, Liver injury, Transaminases increased.
[b] Includes: platelet count decreased, thrombocytopenia
[c] Transient, mild, asymptomatic decrease in platelet counts

6.2 Postmarketing Experience

The following adverse reactions have been identified during post-approval use of ELEVIDYS. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to drug exposure.

Immune System Disorders: Infusion-related reactions, including hypersensitivity reactions and anaphylaxis, have occurred during or up to several hours following ELEVIDYS administration *[see Warnings and Precautions (5.1)]*.

7 DRUG INTERACTIONS

Prior to initiating the corticosteroid regimen required before ELEVIDYS administration, consider the patient's vaccination status. Patients should, if possible, be brought up to date with all immunizations in agreement with current immunization guidelines. Vaccinations should be completed at least 4 weeks prior to initiation of the corticosteroid regimen.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

ELEVIDYS is not intended for use in pregnant women.

In the U.S. general population, the estimated background risks of major birth defects and miscarriage in clinically recognized pregnancies is 2% to 4% and 15% to 20%, respectively.

8.2 Lactation

Risk Summary

There is no information available on the presence of ELEVIDYS in human milk, the effects on the breastfed infant, or the effects on milk production.

8.4 Pediatric Use

The safety and effectiveness of ELEVIDYS for the treatment of Duchenne muscular dystrophy has been established in pediatric patients at least 4 years of age with a confirmed mutation in the *DMD* gene *[see Clinical Pharmacology (12.2), Clinical Studies (14)]*.

8.5 Geriatric Use

The safety and efficacy of ELEVIDYS in geriatric patients with DMD have not been studied.

8.6 Hepatic Impairment

The safety and efficacy of ELEVIDYS in patients with hepatic impairment or elevated GGT have not been studied.

Postpone ELEVIDYS administration in patients with acute liver disease until resolved or controlled. Treatment with ELEVIDYS should be carefully considered in patients with preexisting liver impairment or chronic hepatic viral infection. These patients may be at increased risk of acute serious liver injury *[see Warnings and Precautions (5.2)]*.

In clinical trials, liver function test increase was commonly reported in patients following ELEVIDYS infusion *[see Warnings and Precautions (5.2), Adverse Reactions (6.1)]*.

11 DESCRIPTION

ELEVIDYS (delandistrogene moxeparvovec-rokl) is a recombinant gene therapy designed to deliver the gene encoding the ELEVIDYS micro-dystrophin protein. ELEVIDYS is a non-replicating, recombinant, adeno-associated virus serotype rh74 (AAVrh74) based vector containing the ELEVIDYS micro-dystrophin transgene under the control of the MHCK7 promoter. The genome within the ELEVIDYS AAVrh74 vector contains no viral genes and consequently is incapable of replication or reversion to a replicating form. The micro-dystrophin protein expressed by ELEVIDYS is a shortened version (138 kDa, compared to 427 kDa size of dystrophin expressed in normal muscle cells) that contains selected domains of dystrophin expressed in normal muscle cells.

ELEVIDYS is a preservative-free, sterile, clear, colorless liquid that may have some opalescence and may contain white to off-white particles. ELEVIDYS is a suspension for intravenous infusion with a nominal concentration of 1.33×10^{13} vg/mL and supplied in a single-dose 10 mL vial. Each vial contains an extractable volume of 10 mL and the following excipients: 200mM sodium chloride, 13 mM tromethamine HCl, 7 mM tromethamine, 1mM magnesium chloride, 0.001% poloxamer 188.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

ELEVIDYS is the recombinant gene therapy product that is comprised of a non-replicating, recombinant, adeno-associated virus (AAV) serotype rh74 (AAVrh74) capsid and a single-stranded DNA expression cassette flanked by inverted terminal repeats (ITRs) derived from AAV2. The cassette contains: 1) an MHCK7 gene regulatory component comprising a creatine kinase 7 promoter and an α-myosin heavy chain enhancer, and 2) the DNA transgene encoding the engineered micro-dystrophin protein.

Vector/Capsid: Clinical and nonclinical studies have demonstrated AAVrh74 serotype transduction in skeletal muscle cells. Additionally, in nonclinical studies, AAVrh74 serotype transduction has been demonstrated in cardiac and diaphragm muscle cells.

Promoter: The MHCK7 promoter/enhancer drives transgene expression and has been shown in animal models to drive transgenic micro-dystrophin protein expression predominantly in skeletal muscle (including diaphragm) and cardiac muscle. In clinical studies, muscle biopsy analyses have confirmed micro-dystrophin expression in skeletal muscle.

Transgene: DMD is caused by a mutation in the *DMD* gene resulting in lack of functional dystrophin protein. ELEVIDYS carries a transgene encoding a micro-dystrophin protein consisting of selected domains of dystrophin expressed in normal muscle cells.

ELEVIDYS micro-dystrophin has been demonstrated to localize to the sarcolemma.

12.2 Pharmacodynamics

In 92 patients who received ELEVIDYS in clinical studies, micro-dystrophin protein expression from muscle biopsies (gastrocnemius or biceps brachii) was quantified by western blot and localized by immunofluorescence staining (fiber intensity and percentage micro-dystrophin).

Micro-dystrophin expression (expressed as change from baseline) in ELEVIDYS-treated patients as measured by western blot was the primary objective of Study 1 and Study 2, and a key secondary objective for Study 3. Muscle biopsies were obtained at baseline prior to ELEVIDYS infusion and at Week 12 after ELEVIDYS infusion in all patients. The absolute quantity of micro-dystrophin was measured by western blot assay, adjusted by muscle content and expressed as a percent of control (levels of wild-type dystrophin in patients without DMD or Becker muscular dystrophy) in muscle biopsy samples. Study 1 and 2 results of patients receiving 1.33×10^{14} vg/kg ELEVIDYS are presented in Table 5.

Table 5. Micro-Dystrophin Expression in Study 1 and Study 2 at Week 12 from Baseline (Western Blot Assay)[abc]

Western blot (% of micro-dystrophin compared to control)	Study 1 Part 1 (n=6)	Study 1 Part 2 (n=21)	Study 2 Ambulatory (n=40)	Study 2 Non-ambulatory (n=8)
Mean change from baseline (SD)	43.4 (48.6)	40.7 (32.3)	51.0 (47)	40.1 (35.9)
Median change from baseline (Min, Max)	24.3 (1.6, 116.3)	40.8 (0.0, 92.0)	46.9 (1.9, 197.3)	32.7 (1.4, 116.3)

[a] All patients received 1.33×10^{14} vg/kg, as measured by ddPCR
[b] Change from baseline was statistically significant
[c] Adjusted for muscle content. Control was level of wild-type (normal) dystrophin in normal muscle.

A clear association between Week 12 micro-dystrophin expression and clinical outcome (assessed by change from baseline on the Performance of Upper Limb version 2.0 assessment; Table 7) in non-ambulatory patients has not been established.

In Study 3 Part 1, muscle biopsies were obtained at Week 12 in 31 patients. For the ELEVIDYS-treated patients, the mean micro-dystrophin expression at Week 12 was 34.3% (N=17, SD: 41.0%), compared to placebo patients of 0% (N=14, SD: 0%).

Assessment of micro-dystrophin levels can be meaningfully influenced by differences in sample processing, analytical technique, reference materials, and quantitation methodologies. Therefore, valid comparisons of micro-dystrophin measurements obtained from different assays cannot be made.

12.3 Pharmacokinetics

Vector Distribution and Vector Shedding

Nonclinical Data

Biodistribution of ELEVIDYS was evaluated in tissue samples collected from healthy mice and DMD^{mdx} mice following intravenous administration in toxicology studies. At 12 weeks following ELEVIDYS administration at dose levels of 1.33 ×10^{14} to 4.02 ×10^{14} vg/kg, vector DNA was detected in all major organs with the highest quantities detected in the liver, followed by lower levels in the heart, adrenal glands, skeletal muscle, and aorta. ELEVIDYS was also detected at low levels in the spinal cord, sciatic nerve and gonads (testis). Protein expression of micro-dystrophin was highest in cardiac tissue, exceeding physiologic dystrophin expression levels in healthy mice, with lower levels in the skeletal muscle and diaphragm. In some studies, micro-dystrophin was also detected at low levels in the liver.

Clinical Data

Following IV administration, ELEVIDYS vector genome undergoes distribution via systemic circulation and distributes into target muscle tissues followed by elimination in the urine and feces. ELEVIDYS biodistribution and tissue transduction are detected in the target muscle tissue groups and quantified in the gastrocnemius or biceps femoris biopsies obtained from patients with mutations in the *DMD* gene. Evaluation of ELEVIDYS vector genome exposure in clinical muscle biopsies at Week 12 post-dose expressed as copies per nucleus revealed ELEVIDYS drug distribution and transduction with a mean change from baseline of 2.91 and 3.44 copies per nucleus at the recommended dose of 1.33 × 10^{14} vg/kg for Study 1 and Study 2 Cohort 1, respectively.

In Study 2 Cohorts 1-3, the biodistribution and vector shedding of ELEVIDYS in the serum and excreta were quantified, respectively. The mean maximum concentration (C_{max}) in the serum was 0.0055 × 10^{13} copies/mL and 2.78 × 10^{6} copies/mL in the urine, 7.86 × 10^{7} copies/mL in the saliva, and 4.87 × 10^{7} copies/µg in the feces. The median time to achieve maximum concentration (T_{max}) was 5.8 hours post-dose in the serum, followed by 6.7 hours, 6.5 hours and 13 days post-dose in the saliva, urine, and feces, respectively. The median time to achieve first below limit of quantification (BLOQ) sample followed by 2 consecutive BLOQ samples was 55 days post-dose for serum. The median time to achieve complete elimination as the first below limit of detection (BLOD) sample followed by 2 consecutive BLOD samples were 49.8 days, 78 days and 162 days post-dose for saliva, urine and feces, respectively. The estimated elimination half-life of ELEVIDYS vector genome in the serum is approximately 12 hours, and the majority of the drug is expected to be cleared from the serum by 1-week post-dose. In the excreta, the estimated elimination half-life of ELEVIDYS vector genome is approximately 40 hours, 55 hours, and 60 hours in the urine, feces, and saliva, respectively. As an AAV-based gene therapy that consists of a protein capsid containing the transgene DNA genome of interest, ELEVIDYS capsid proteins are broken down through proteasomal degradation following AAV entry into target cells. As such, ELEVIDYS is not likely to exhibit the drug-drug interaction potential mediated by known drug metabolizing enzymes (cytochrome P450-based) and drug transporters.

12.6 Immunogenicity

The observed incidence of anti-AAVrh74 antibodies is highly dependent on the sensitivity and specificity of the assay. Differences in assay methods preclude meaningful comparisons of the incidence of anti-AAVrh74 antibodies in the studies described below with the incidence of anti-AAVrh74 antibodies in other studies.

In ELEVIDYS clinical studies, patients were required to have baseline anti-AAVrh74 total binding antibodies of ≤1:400, measured using an investigational total binding antibody enzyme-linked immunosorbent assay (ELISA), and only patients with baseline anti-AAVrh74 total binding antibodies <1:400 were enrolled in those studies. The safety and efficacy of ELEVIDYS in patients with elevated anti-AAVrh74 total binding antibody titer (≥1:400) have not been evaluated *[see Clinical Studies (14)]*.

Across clinical studies evaluating a total of 156 patients, elevated anti-AAVrh74 total binding antibodies titers were observed in all patients following a one-time ELEVIDYS infusion. Anti-AAVrh74 total binding antibody

titers reached at least 1:102,400 in every patient, and the maximum titers exceeded 1:26,214,400 in certain patients. The safety of re-administration of ELEVIDYS or any other AAVrh74 vector-based gene therapy in the presence of high anti-AAVrh74 total binding antibody titer has not been evaluated in humans *[see Warnings and Precautions (5.5)]*.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No animal studies have been performed to evaluate the effects of ELEVIDYS on carcinogenicity, mutagenesis, or impairment of fertility.

14 CLINICAL STUDIES

The efficacy of ELEVIDYS was evaluated in two double-blind, placebo-controlled studies (Study 1 [NCT 03769116] and Study 3 [NCT 05096221]) and one open-label study (Study 2 [NCT 04626674]) in which a total of 214 male patients with a confirmed disease-causing mutation in the *DMD* gene were dosed.

Study 1

Study 1 is a completed multi-center study including:

- Part 1: a 48-week, randomized, double-blind, placebo-controlled period
- Part 2: a 48-week period that began following completion of Part 1. Patients who received placebo during Part 1 were treated with ELEVIDYS, and patients treated with ELEVIDYS during Part 1 received placebo.

The study population consisted of male ambulatory DMD patients (N=41) aged 4 through 7 years with either a confirmed frameshift mutation, or a premature stop codon mutation between exons 18 to 58 in the *DMD* gene.

Patients were randomized 1:1 to receive either ELEVIDYS (N=20) or placebo (N=21), as a single intravenous infusion via a peripheral limb. Randomization was stratified by age (i.e., aged 4 to 5 years vs. aged 6 to 7 years). In the 4 through 5-year-old subgroup, the mean age, mean weight and mean NSAA total score (range) for the ELEVIDYS-treated patients (n=8) were 4.98 years, 20.1 kg and 20.1 (17-23), and for the placebo patients (n=8) were 5.15 years, 19.8 kg and 20.4 (15-24). In the ELEVIDYS group, eight patients received 1.33×10^{14} vg/kg of ELEVIDYS, and 12 patients received lower doses. Key demographic and baseline characteristics are presented in Table 6.

Table 6: Key Demographic and Baseline Characteristics (Study 1 Part 1)

Characteristic	All (n=41)	ELEVIDYS (n=20)	Placebo (n=21)
Race (%) Asian/Black or African American/White/Other	12/0/73/15	20/0/65/15	5/0/81/14
Ethnicity (%) Hispanic or Latino/ Other	12/88	5/95	19/81
Mean age [range] (years)	6.3 [4.3 to 7.9]	6.3 [4.5 to 7.9]	6.2 [4.3 to 7.9]
Mean weight [range] (kg)	22.4 [15.0 to 34.5]	23.3 [18.0 to 34.5]	21.6 [15.0 to 30.0]
Mean NSAA total score [range]	21.2 [13 to 29]	19.8 [13 to 26]	22.6 [15 to 29]
Mean time to rise from floor [range] (seconds)	4.3 [2.7 to 10.4]	5.1 [3.2 to 10.4]	3.6 [2.7 to 4.8]

All patients were on a stable dose of corticosteroids for DMD for at least 12 weeks prior to ELEVIDYS infusion. All randomized patients had baseline anti-AAVrh74 antibody titers <1:400 as determined by an investigational total binding antibody ELISA.

One day prior to treatment with ELEVIDYS or placebo, the patient's background dose of corticosteroid for DMD was increased to at least 1 mg/kg of a corticosteroid (prednisone equivalent) daily and was continued at this level for at least 60 days after the infusion, unless earlier tapering was clinically indicated.

The efficacy outcomes of Study 1 were to evaluate expression of micro-dystrophin in skeletal muscle, and to evaluate the effect of ELEVIDYS on the North Star Ambulatory Assessment (NSAA) total score.

Results of micro-dystrophin measured by western blot are presented in Table 5 *[see Clinical Pharmacology (12.2)]*.

The change in NSAA total score was assessed from baseline to Week 48 after infusion of ELEVIDYS or placebo. The difference between the ELEVIDYS and placebo groups was not statistically significant (p=0.37). The least squares (LS) mean changes in NSAA total score from baseline to Week 48 was 1.7 (standard error [SE]: 0.6) points for the ELEVIDYS group and 0.9 (SE: 0.6) points for the placebo group.

Exploratory subgroup analyses showed that for patients aged 4 through 5 years, the LS mean changes (SE) in NSAA total score from baseline to Week 48 were 4.3 (0.7) points for the ELEVIDYS group, and 1.9 (0.7) points for the placebo group, a numerical advantage for ELEVIDYS. For patients aged 6 through 7 years, the LS mean changes (SE) in NSAA total score from baseline to Week 48 were -0.2 (0.7) points for the ELEVIDYS group and 0.5 (0.7) points for the placebo group, a numerical disadvantage for ELEVIDYS.

Study 2

Study 2 is an ongoing, open-label, multi-center study which includes 5 cohorts of 48 male DMD patients.

Patients in cohorts 1, 2 and 3 have a confirmed frameshift, splice site or premature stop codon mutation anywhere in the *DMD* gene, while patients in cohort 4 included patients with mutations in the *DMD* gene starting at or after exon 18. All patients in cohort 5 had mutations that partially or fully overlap with exons 1-17 in the *DMD* gene. Patients received corticosteroids for DMD before infusion according to Table 1 *[see Dosage and Administration (2.2)]*. All patients had baseline anti-AAVrh74 antibodies titers ≤1:400 as determined by the investigational total binding antibody ELISA. Patients received a single intravenous infusion of 1.33×10^{14}

vg/kg ELEVIDYS if they weighed less than 70 kg or 9.31×10^{15} vg/kg total fixed dose if they weighed 70 kg or greater.

Cohorts 1, 2, 4 and 5a enrolled 40 ambulatory patients 3 to 12 years of age, with weights ranging from 12.5 to 50.5 kg, baseline mean NSAA total score of 20.3 (11 to 30), and mean time to rise from floor of 4.7 seconds (2.4 to 9.7). Cohorts 3 and 5b include 8 non-ambulatory patients 10 to 20 years of age, with weights ranging from 36.1 to 80.1 kg. Overall key demographics and key baseline characteristics by Cohort are presented in Table 7.

Table 7: Key Demographic and Baseline Characteristics for Study 2

Characteristics	All (n=48)	Cohort 1 (n=20)	Cohort 2 (n=7)	Cohort 3[a] (n=6)	Cohort 4 (n=7)	Cohort 5a (n=6)	Cohort 5b[a] (n=2)
Race (%) Asian/Black or African American/White/Other	8/6/77/8	5/5/75/15	14/0/71/14	0/0/100/0	14/0/86/0	0/33/67/0	50/0/50/0
Ethnicity (%) Hispanic or Latino/ Not Hispanic or Latino	15/85	25/75	14/86	0/100	14/86	0/100	0/100
Mean age [range] (years)	7.7 [3.2 to 20.2]	5.8 [4.4 to 7.9]	10.1 [8.0 to 12.1]	15.3 [9.9 to 20.2]	3.5 [3.2 to 3.9]	6.7 [4.7 to 8.6]	13.4 [12.3 to 14.6]
Mean weight [range] (kg)	30.1 [12.5 to 80.1]	21.2 [15.2 to 33.1]	37.1 [28.0 to 50.5]	59.9 [36.1 to 80.1]	15.2 [12.5 to 16.5]	32.1 [19.1 to 47.4]	51.2 [43.4 to 59.0]
Mean NSAA total score [range]	20.3 [11 to 30]	22.1 [18 to 26]	20.7 [17 to 26]	N/A	12.9 [11 to 17]	22.5 [18 to 30]	N/A
Mean time to rise from floor [range] (seconds)	4.7 [2.4 to 9.7]	4.2 [2.4 to 8.2]	5.9 [3.8 to 9.7]	N/A	5.2 [3.8 to 6.7]	4.6 [2.5 to 7.7]	N/A
Mean Performance of Upper Limb v. 2.0 score [range]	30.7 [18 to 42]	NA	38.9 [33 to 42]	22.2 [18 to 31]	NA	NA	27.5 [21 to 34]

[a] NSAA and Time to rise from floor were not evaluated in non-ambulatory patients

The efficacy outcome measure of the study was to evaluate the effect of micro-dystrophin expression as measured by western blot. Results are presented in Table 5 *[see Clinical Pharmacology (12.2)]*.

Study 3

Study 3 is a multi-center, randomized, double-blind, placebo-controlled study in which 125 ambulatory male patients aged 4 through 7 years, with a confirmed frameshift, splice site, premature stop codon, or other disease-causing mutation in the *DMD* gene starting at or after exon 18, were dosed. Patients with exon 45 (inclusive), or in-frame deletions, in-frame duplications, and variants of uncertain significance ("VUS"), were excluded. Patients received corticosteroids for DMD before infusion according to Table 1 *[see Dosage and Administration (2.2)]*. All patients had baseline anti-AAVrh74 antibodies titers <1:400 as determined by the investigational total binding antibody ELISA and received a single intravenous infusion of 1.33×10^{14} vg/kg ELEVIDYS. Key demographic and baseline characteristics are presented in Table 8.

The efficacy outcome measure of the study was to evaluate the effect of ELEVIDYS on physical function as assessed by the NSAA total score. Key secondary outcome measures were to evaluate expression of micro-dystrophin in skeletal muscle, time to rise from floor, and time of 10-meter walk/run. Additional efficacy outcome measures included time of 100-meter walk/run, and time to ascend 4 steps. Results of micro-dystrophin measured by western blot are presented in Table 5 *[see Clinical Pharmacology (12.2)]*.

Table 8: Key Demographic and Baseline Characteristics for Study 3

Characteristic	ELEVIDYS (n=63)	Placebo (n=62)
Race (%) Asian/Black or African American/ White/Multiple/Other/Not Reported	13/0/78/2/3/5	18/3/74/0/2/3
Ethnicity (%) Hispanic or Latino/Not Hispanic or Latino/ Not Reported/Unknown	24/75/0/2	13/86/2/0
Mean age [range] (years)	6.0 [4.1 to 7.9]	6.1 [4.0 to 7.9]
Mean weight [range] (kg)	21.3 [13.5 to 38.5]	22.4 [14.4 to 41.6]
Mean NSAA total score [range]	23.10 [14 to 32]	22.82 [15.5 to 30]
Mean time to rise from floor [range] (seconds)	3.52 [1.9 to 5.8]	3.60 [2.3 to 5]
Mean time of 10-meter walk/run [range] (seconds)	4.82 [3.2 to 6.9]	4.92 [3.7 to 7]
Mean time of 100-meter walk/run [range] (seconds)	60.67 [38.0 to 129.2]	63.01 [38.7 to 118.1]
Mean time to ascend 4 steps [range] (seconds)	3.17 [1.6 to 7.1]	3.37 [1.5 to 7.1]

The change in NSAA total score was assessed from baseline to Week 52 after infusion of ELEVIDYS or placebo. The difference between the ELEVIDYS (n=63) and placebo groups (n=61) was not statistically significant (p=0.24). The least squares (LS) mean changes in NSAA total score from baseline to Week 52 was 2.57 (95% confidence interval [CI]: 1.80, 3.34) points for the ELEVIDYS group and 1.92 (95% CI: 1.14, 2.70) points for the placebo group, with a LS mean difference from placebo of 0.65 (95% CI: -0.45, 1.74). Changes of clinical relevance were noted in three secondary efficacy endpoints, including time to rise from the floor, 10-meter walk/run and time to ascend 4 steps.

Table 9: Change from Baseline to Week 52 of Timed Function Tests in Study 3 Part 1

	ELEVIDYS	Placebo	LS Mean Difference from placebo (95% CI)
Time to rise from the floor (seconds)	n=63	n=61	-
LS mean Change (95% CI)	-0.27 (-0.56, 0.02)	0.37 (0.08, 0.67)	-0.64 (-1.06, -0.23)
Time of 10-meter walk/run (seconds)	n=63	n=61	-
LS mean Change (95% CI)	-0.34 (-0.55, -0.14)	0.08 (-0.13, 0.29)	-0.42 (-0.71, -0.13)
Time of 100-meter walk/run (seconds)	n=59	n=57	-
LS mean Change (95% CI)	-6.57 (-10.05, -3.09)	-3.28 (-6.86, 0.29)	-3.29 (-8.28, 1.70)
Time to ascend 4 steps (seconds)	n=62	n=60	-
LS mean Change (95% CI)	-0.44 (-0.69, -0.20)	-0.08 (-0.33, 0.17)	-0.36 (-0.71, -0.01)

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

ELEVIDYS is shipped frozen (≤ -60ºC [-76ºF]) in 10 mL vials.

ELEVIDYS is supplied as a customized kit to meet dosing requirements for each patient *[see Dosage and Administration (2.1)]*. Each kit contains:

- Ten (10) to seventy (70) single-dose vials of ELEVIDYS
- One alcohol wipe per vial

Each ELEVIDYS pack may contain a maximum of two different drug product lots.

The total number of vials in each kit corresponds to the dosing requirement for the individual patient, based on the patient's body weight, and is specified on the package *[see Dosage and Administration (2.2)]*. Each kit includes a specified number of ELEVIDYS vials (with a minimum of 10 vials for a patient with 10.0 – 10.4 kg body weight range, and a maximum of 70 vials for a patient with body weight of 69.5 kg and above).
Kit sizes and National Drug Codes (NDC) are provided in Table 10.

Table 10: ELEVIDYS Multi-vial Kits

Patient Weight (kg)	Total Vials per Kit	Total Dose Volume per Kit (mL)	NDC Number
10.0 – 10.4	10	100	60923-501-10
10.5 – 11.4	11	110	60923-502-11
11.5 – 12.4	12	120	60923-503-12
12.5 – 13.4	13	130	60923-504-13
13.5 – 14.4	14	140	60923-505-14
14.5 – 15.4	15	150	60923-506-15
15.5 – 16.4	16	160	60923-507-16
16.5 – 17.4	17	170	60923-508-17
17.5 – 18.4	18	180	60923-509-18
18.5 – 19.4	19	190	60923-510-19
19.5 – 20.4	20	200	60923-511-20
20.5 – 21.4	21	210	60923-512-21
21.5 – 22.4	22	220	60923-513-22
22.5 – 23.4	23	230	60923-514-23
23.5 – 24.4	24	240	60923-515-24
24.5 – 25.4	25	250	60923-516-25
25.5 – 26.4	26	260	60923-517-26

Patient Weight (kg)	Total Vials per Kit	Total Dose Volume per Kit (mL)	NDC Number
26.5 – 27.4	27	270	60923-518-27
27.5 – 28.4	28	280	60923-519-28
28.5 – 29.4	29	290	60923-520-29
29.5 – 30.4	30	300	60923-521-30
30.5 – 31.4	31	310	60923-522-31
31.5 – 32.4	32	320	60923-523-32
32.5 – 33.4	33	330	60923-524-33
33.5 – 34.4	34	340	60923-525-34
34.5 – 35.4	35	350	60923-526-35
35.5 – 36.4	36	360	60923-527-36
36.5 – 37.4	37	370	60923-528-37
37.5 – 38.4	38	380	60923-529-38
38.5 – 39.4	39	390	60923-530-39
39.5 – 40.4	40	400	60923-531-40
40.5 – 41.4	41	410	60923-532-41
41.5 – 42.4	42	420	60923-533-42
42.5 – 43.4	43	430	60923-534-43
43.5 – 44.4	44	440	60923-535-44
44.5 – 45.4	45	450	60923-536-45
45.5 – 46.4	46	460	60923-537-46
46.5 – 47.4	47	470	60923-538-47
47.5 – 48.4	48	480	60923-539-48
48.5 – 49.4	49	490	60923-540-49
49.5 – 50.4	50	500	60923-541-50
50.5 – 51.4	51	510	60923-542-51
51.5 – 52.4	52	520	60923-543-52
52.5 – 53.4	53	530	60923-544-53
53.5 – 54.4	54	540	60923-545-54
54.5 – 55.4	55	550	60923-546-55

Patient Weight (kg)	Total Vials per Kit	Total Dose Volume per Kit (mL)	NDC Number
55.5 – 56.4	56	560	60923-547-56
56.5 – 57.4	57	570	60923-548-57
57.5 – 58.4	58	580	60923-549-58
58.5 – 59.4	59	590	60923-550-59
59.5 – 60.4	60	600	60923-551-60
60.5 – 61.4	61	610	60923-552-61
61.5 – 62.4	62	620	60923-553-62
62.5 – 63.4	63	630	60923-554-63
63.5 – 64.4	64	640	60923-555-64
64.5 – 65.4	65	650	60923-556-65
65.5 – 66.4	66	660	60923-557-66
66.5 – 67.4	67	670	60923-558-67
67.5 – 68.4	68	680	60923-559-68
68.5 – 69.4	69	690	60923-560-69
69.5 and above	70	700	60923-561-70

A 10 mL single-dose vial carton for ELEVIDYS (NDC 60923-562-01) is not sold individually.

16.2 Storage and Handling

- ELEVIDYS is shipped and delivered at ≤ −60ºC [−76ºF].
- ELEVIDYS can be refrigerated for up to 14 days when stored at 2°C to 8°C (36º F to 46º F) in the upright position.
- Do not refreeze.
- Do not shake.
- Do not place back in the refrigerator once brought to room temperature.
- Follow local guidelines on handling of biological waste.

17 PATIENT COUNSELING INFORMATION

Inform patients or caregivers that:

- Infusion-related reactions including hypersensitivity and anaphylaxis have occurred during and after ELEVIDYS infusion. Possible symptoms of infusion-related reactions are fast heart rate, fast breathing, swollen lips, being short of breath, nostrils widening, hives, red and blotchy skin, itchy or inflamed lips, rash, vomiting, nausea, chills and fever. Contact a healthcare provider immediately if the patient experiences such a reaction *[see Warnings and Precautions (5.1)]*.
- ELEVIDYS can increase certain liver enzyme levels and cause acute serious liver injury. Patients will receive oral corticosteroid medication before and after infusion with ELEVIDYS. Weekly blood tests will

be required to monitor liver enzyme levels for 3 months after treatment. Contact a healthcare provider immediately if the patient's skin and/or whites of the eyes appear yellowish, or if the patient misses a dose of corticosteroid or vomits it up *[see Warnings and Precautions (5.2)]*.

- Immune-mediated myositis (an immune response affecting muscles) was observed in patients with a deletion mutation in the *DMD* gene that is contraindicated. Contact a physician immediately if the patient experiences any unexplained increased muscle pain, tenderness, or weakness, including difficulty swallowing, difficulty breathing or difficulty speaking, as these may be symptoms of myositis *[see Warnings and Precautions (5.3)]*.
- Myocarditis (inflammation of the heart) has been observed within days following ELEVIDYS infusion. Weekly monitoring of troponin-I for the first month after treatment is required. Contact a healthcare provider immediately if the patient begins to experience chest pain and/or shortness of breath *[see Warnings and Precautions (5.4)]*.
- Patient's immunizations should be up to date with current immunization guidelines prior to initiation of the corticosteroid regimen required before ELEVIDYS infusion. Vaccinations should be completed at least 4 weeks prior to initiation of the corticosteroid regimen *[see Drug Interactions (7)]*.
- Due to the concomitant administration of corticosteroids, an infection (e.g., cold, flu, gastroenteritis, otitis media, bronchiolitis, etc.) before or after ELEVIDYS infusion could lead to more serious complications. Contact a healthcare provider immediately if symptoms suggestive of infection are observed (e.g., coughing, wheezing, sneezing, runny nose, sore throat, or fever).
- Vector shedding of ELEVIDYS occurs primarily through body waste. Practice proper hand hygiene, such as hand washing, when coming into direct contact with patient body waste. Place potentially contaminated materials that may have the patient's bodily fluids/waste in a sealable bag and dispose into regular trash. These precautions should be followed for one month after ELEVIDYS infusion.

Manufactured for: Sarepta Therapeutics, Inc.
Cambridge, MA 02142 USA
U.S. license number 2308

Exhibit D

U.S. FOOD & DRUG ADMINISTRATION

Our STN: BL 125781/0

ACCELERATED BLA APPROVAL
June 22, 2023

Sarepta Therapeutics, Inc.
Attention: Patrick O'Malley
215 First Street
Cambridge, MA 02142

Dear Mr. O'Malley:

Please refer to your Biologics License Application (BLA) received September 28, 2022, under section 351(a) of the Public Health Service Act (PHS Act) for delandistrogene moxeparvovec-rokl.

LICENSING

We are issuing Department of Health and Human Services U.S. License No. 2308 to Sarepta Therapeutics, Inc., Cambridge, MA, under the provisions of section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products and pursuant to section 506(c) of the Federal Food, Drug, and Cosmetic Act (FDCA) and the regulations for accelerated approval, 21 CFR 601.41. The license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license you are authorized to manufacture the product delandistrogene moxeparvovec-rokl. Delandistrogene moxeparvovec-rokl is indicated for treatment of ambulatory pediatric patients aged 4 through 5 years with Duchenne muscular dystrophy (DMD) with a confirmed mutation in the *DMD* gene.

The review of this product was associated with the following National Clinical Trial (NCT) numbers: NCT03375164, NCT03769116, and NCT04626674.

ACCELERATED APPROVAL REQUIREMENTS

Under accelerated approval statutory provisions and regulations, we may grant marketing approval for a biological product on the basis of adequate and well-controlled clinical trials establishing that the biological product has an effect on a surrogate endpoint that is reasonably likely, based on epidemiologic, therapeutic, pathophysiologic, or other evidence, to predict clinical benefit, or on the basis of an effect on a clinical endpoint other than survival or irreversible morbidity that is reasonably likely to predict survival or irreversible morbidity. This approval requires you

to study the biological product further, to verify and describe its clinical benefit, where there is uncertainty as to the relation of the surrogate endpoint to clinical benefit, or of the observed clinical benefit to ultimate outcome.

Approval under these statutory provisions and regulations requires, among other things, that you conduct adequate and well-controlled clinical trials to verify and describe clinical benefit attributable to this product. Clinical benefit is evidenced by effects such as improved North Star Ambulatory Assessment (NSAA) Total Score from baseline to Week 52 after treatment with delandistrogene moxeparvovec-rokl when compared with an appropriate concurrent control group.

Accelerated Approval Required Studies

We remind you of your postmarketing requirement specified in your submission of June 6, 2023.

1. Complete Study SRP-9001-301 Part 1, an ongoing, randomized, double-blinded clinical trial intended to describe and verify clinical benefit of delandistrogene moxeparvovec-rokl in ambulatory patients with Duchenne muscular dystrophy (DMD). The trial evaluates the primary endpoint of North Star Ambulatory Assessment (NSAA) and compares delandistrogene moxeparvovec-rokl to placebo in 125 ambulatory patients with DMD with confirmed mutation in the *DMD* gene.

   Final Protocol Submission: Submitted

   Trial Completion: September 30, 2023

   Final Report Submission: January 31, 2024

We expect you to complete and report this trial within the framework described in your letter of June 6, 2023.

We acknowledge that you have provided the final protocol to your IND 17763. Please provide a letter of cross-reference to this BLA, STN BL 125781, explaining that this protocol was submitted to the IND. Please refer to the sequential number for each trial and the submission number as shown in this letter.

You must conduct this trial with due diligence. If this required postmarketing trial fails to verify that clinical benefit is conferred by delandistrogene moxeparvovec-rokl or is not conducted with due diligence, including with respect to the conditions set forth below, we may withdraw this approval.

You must submit reports of the progress of each trial required under section 506(c) of the FDCA to this BLA 180 days after the date of approval of this BLA and every 180

days thereafter as required under section 506B(a)(2) of the FDCA. The submission of these reports will be subject to a 60-day grace period.

The reports should include:

- expected trial completion and final report submission dates
- any changes in plans since the last report, with rationale for any changes,
- the current number of patients entered into each trial.

Reports submitted 180 days after the date of approval of this BLA, subject to a 60-day grace period, and on such date each year thereafter must be labeled **180-Day AA PMR Progress Report**.

Reports submitted one year after the date of approval of this BLA and on such date each year thereafter may be submitted as part of your annual status report required under section 506B(a)(1) of the FDCA and 21 CFR 601.70. FDA will consider the submission of your annual status report under section 506B(a)(1) and 21 CFR 601.70, in addition to the submission of progress reports 180 days after the date of approval and on such date each year thereafter, to satisfy the periodic reporting requirement under section 506B(a)(2).

Label your annual report as an **Annual Status Report of Postmarketing Requirements/Commitments** and submit it to the FDA each year within 60 calendar days of the anniversary date of this letter until all Postmarketing Requirements and 506B Commitments are fulfilled or released.

Please submit a final study report as a supplement to this BLA, STN BL 125781. For administrative purposes, all submissions related to this postmarketing study requirement must be clearly designated as "**Subpart E Postmarketing Study Requirements.**"

MANUFACTURING LOCATIONS

Under this license, you are approved to manufacture delandistrogene moxeparvovec-rokl drug substance at Catalent Pharma Services, Catalent Maryland (BWI), 7555 Harmans Road, Harmans, MD 20177, USA. The final formulated drug product will be manufactured at Catalent Pharma Solutions, Catalent Maryland (Biopark), 801 West Baltimore Street, Suite 302, Baltimore, MD 21201, USA; and labeled and packaged at the (b) (4)

You may label your product with the proprietary name ELEVIDYS and market it in 10 mL vials.

DATING PERIOD

The dating period for delandistrogene moxeparvovec-rokl shall be 12 months from the date of manufacture when stored at ≤ -60°C. The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. Following the final sterile filtration, no reprocessing/reworking is allowed without prior approval from the Agency. The dating period for your drug substance shall be (b) (4) when stored at (b) (4). We have approved the stability protocols in your license application for the purpose of extending the expiration dating period of your drug substance and drug product under 21 CFR 601.12.

FDA LOT RELEASE

Please submit protocols showing results of all applicable tests. You may not distribute any lots of product until you receive a notification of release from the Director, Center for Biologics Evaluation and Research (CBER).

BIOLOGICAL PRODUCT DEVIATIONS

You must submit reports of biological product deviations under 21 CFR 600.14. You should identify and investigate all manufacturing deviations promptly, including those associated with processing, testing, packaging, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on FORM FDA 3486 to the Director, Office of Compliance and Biologics Quality, electronically through the eBPDR web application or at the address below. Links for the instructions on completing the electronic form (eBPDR) may be found on CBER's web site at https://www.fda.gov/vaccines-blood-biologics/report-problem-center-biologics-evaluation-research/biological-product-deviations.

Food and Drug Administration
Center for Biologics Evaluation and Research
Document Control Center
10903 New Hampshire Ave.
WO71-G112
Silver Spring, MD 20993-0002

MANUFACTURING CHANGES

You must submit information to your BLA for our review and written approval under 21 CFR 601.12 for any changes in, including but not limited to, the manufacturing, testing, packaging or labeling of delandistrogene moxeparvovec-rokl, or in the manufacturing facilities.

LABELING

We hereby approve the draft content of labeling including the Package Insert submitted under amendment 76, dated June 21, 2023, and the draft carton and container labels submitted under amendments 70 and 77, dated June 15, 2023 and June 21, 2023.

CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, please submit the final content of labeling (21 CFR 601.14) in Structured Product Labeling (SPL) format via the FDA automated drug registration and listing system, (eLIST) as described at http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm. Content of labeling must be identical to the Package Insert submitted on June 21, 2023. Information on submitting SPL files using eLIST may be found in the guidance for industry *SPL Standard for Content of Labeling Technical Qs and As* at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072392.pdf.

The SPL will be accessible via publicly available labeling repositories.

CARTON AND CONTAINER LABELS

Please electronically submit final printed carton and container labels identical to the carton and container labels submitted on June 15, 2023 and June 21, 2023, according to the guidance for industry *Providing Regulatory Submissions in Electronic Format — Certain Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications* at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/providing-regulatory-submissions-electronic-format-certain-human-pharmaceutical-product-applications.

All final labeling should be submitted as Product Correspondence to this BLA, STN BL 125781 at the time of use and include implementation information on Form FDA 356h.

PROMOTIONAL MATERIALS

Please note that the accelerated approval regulation concerning promotional materials (21 CFR 601.45) stipulates that all advertising and promotional labeling items that you wish to distribute in the first 120 days following approval, must have been received by FDA prior to the approval date. After approval, promotional items intended for dissemination after the first 120 days following approval must be submitted to the FDA at least 30 days prior to the anticipated distribution date. Please submit draft materials with a cover letter noting that the items are for accelerated approval, and an accompanying FORM FDA 2253 to the Advertising and Promotional Labeling Branch at the following address:

Food and Drug Administration
Center for Biologics Evaluation and Research
Document Control Center
10903 New Hampshire Ave.
WO71-G112
Silver Spring, MD 20993-0002

You must submit copies of your final advertisement and promotional labeling at the time of initial dissemination or publication, accompanied by FORM FDA 2253 (21 CFR 601.12(f)(4)).

Alternatively, you may submit promotional materials for accelerated approval products electronically in eCTD format. For more information about submitting promotional materials in eCTD format, see the draft guidance for industry *Providing Regulatory Submissions in Electronic and Non-Electronic Format—Promotional Labeling and Advertising Materials for Human Prescription Drugs* at https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM443702.pdf.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence or substantial clinical experience to support such claims (21 CFR 202.1(e)(6)).

ADVERSE EVENT REPORTING

You must submit adverse experience reports in accordance with the adverse experience reporting requirements for licensed biological products (21 CFR 600.80) and you must submit distribution reports as described in 21 CFR 600.81. In addition, to the reporting requirements in 21 CFR 600.80, you must submit adverse experience reports for acute liver injury, immune-mediated myositis, myocarditis, and thrombotic microangiopathy as 15-day expedited reports (regardless of seriousness or expectedness) to the FDA Adverse Event Reporting System (FAERS). Acute liver injury, immune-mediated myositis, myocarditis, and thrombotic microangiopathy reports must be submitted as 15-day expedited reports for 3 years following the date of product licensure. For information on adverse experience reporting, please refer to the guidance for industry *Providing Submissions in Electronic Format —Postmarketing Safety Reports* at https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072369 and FDA's Adverse Event reporting System website at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/ucm115894.htm. For information on distribution reporting, please refer to the guidance for industry *Electronic Submission of Lot Distribution Reports* at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Post-MarketActivities/LotReleases/ucm061966.htm.

RARE PEDIATRIC DISEASE PRIORITY REVIEW VOUCHER

We also inform you that you have been granted a rare pediatric disease priority review voucher (PRV), as provided under section 529 of the FDCA. This PRV has been assigned a tracking number, PRV BLA 125781. All correspondences related to this voucher should refer to this tracking number.

This voucher entitles you to designate a single human drug application submitted under section 505(b)(1) of the FDCA or a single biologic application submitted under section 351 of the Public Health Service Act as qualifying for a priority review. Such an application would not have to meet any other requirements for a priority review. The list below describes the sponsor responsibilities and the parameters for using and transferring a rare pediatric disease priority review voucher.

- The sponsor who redeems the PRV must notify FDA of its intent to submit an application with a PRV at least 90 days before submission of the application and must include the date the sponsor intends to submit the application. This notification should be prominently marked, "**Notification of Intent to Submit an Application with a Rare Pediatric Disease Priority Review Voucher**."
- This PRV may be transferred, including by sale, by you to another sponsor of a human drug or biologic application. There is no limit on the number of times that the PRV may be transferred, but each person to whom the PRV is transferred must notify FDA of the change in ownership of the voucher not later than 30 days after the transfer. If you retain and redeem this PRV, you should refer to this letter as an official record of the voucher. If the PRV is transferred, the sponsor to whom the PRV has been transferred should include a copy of this letter (which will be posted on our website as are all approval letters) and proof that the PRV was transferred.
- FDA may revoke the PRV if the rare pediatric disease product for which the PRV was awarded is not marketed in the U.S. within 1 year following the date of approval.
- The sponsor of an approved rare pediatric disease product application who is awarded a PRV must submit a report to FDA no later than 5 years after approval that addresses, for each of the first 4 post-approval years:
    - the estimated population in the U.S. suffering from the rare pediatric disease for which the product was approved (both the entire population and the population aged 0 through 18 years),
    - the estimated demand in the U.S. for the product, and
    - the actual amount of product distributed in the U.S.

You may also review the requirements related to this program by visiting FDA's Rare Pediatric Disease PRV Program webpage available at https://www.fda.gov/ForIndustry/DevelopingProductsforRareDiseasesConditions/RarePediatricDiseasePriorityVoucherProgram/default.htm.

PEDIATRIC REQUIREMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because the biological product for this indication has an orphan drug designation, you are exempt from this requirement.

POSTMARKETING COMMITMENTS SUBJECT TO REPORTING REQUIREMENTS UNDER SECTION 506B

We acknowledge your written commitment as described in your submission of June 22, 2023, as outlined below:

2. Sarepta commits to conducting adequate analytical and clinical validation testing to establish an (b) (4) that can be used to identify patients with DMD who may benefit from delandistrogene moxeparvovec-rokl therapy. The results of the validation study are intended to inform product labeling. The clinical validation should be supported by a clinical bridging study comparing the in (b) (4) and the clinical trial enrollment assays.

   (b) (4)

The PMC will be considered fulfilled (b) (4)

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. Label your annual report as an **Annual Status Report of Postmarketing Requirements/Commitments** and submit it to the FDA each year within 60 calendar days of the anniversary date of this letter until all Requirements and Commitments subject to the reporting requirements of section 506B of the FDCA are fulfilled or released. The status report for each study should include:

- the sequential number for each study as shown in this letter;
- information to identify and describe the postmarketing commitment;
- the original schedule for the commitment;

- the status of the commitment (i.e., pending, ongoing, delayed, terminated, or submitted); and,
- an explanation of the status including, for clinical studies, the patient accrual rate (i.e., number enrolled to date and the total planned enrollment).

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our website at https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Post-marketingPhaseIVCommitments/default.htm.

POSTMARKETING COMMITMENTS NOT SUBJECT TO THE REPORTING REQUIREMENTS UNDER SECTION 506B

We acknowledge your written commitments as described in your submission of May 30, 2023, and June 8, 2023, as outlined below:

3. Sarepta Therapeutics, Inc. commits to performing (b) (4) as a "Postmarketing Commitment - Final Study Report" by July 31, 2024.

   Final Report Submission: July 31, 2024

4. Sarepta Therapeutics, Inc. commits to submitting a final report for the supplemental (b) (4) manufacturing runs for (b) (4) at the Catalent facility as a "Postmarketing Commitment - Final Study Report" by June 30, 2024.

   Final Report Submission: June 30, 2024

5. Sarepta Therapeutics, Inc. commits to submitting a final report of the (b) (4) as a "Postmarketing Commitment - Final Study Report" by March 31, 2024.

   Final Report Submission: March 31, 2024

6. Sarepta Therapeutics, Inc. commits to revising the system suitability criteria set in the SOP for (b) (4) to reflect the assay variability (percent coefficient of variation; %CV) observed in intermediate precision during assay validation and to submitting the revised SOP as a "Postmarketing Commitment - Final Study Report" by December 31, 2023.

   Final Report Submission: December 31, 2023

7. Sarepta Therapeutics, Inc. commits to revising the system suitability in the SOP for the in (b) (4) assay to include a parameter determining (b) (4)

and to submitting the revised SOP as a "Postmarketing Commitment – Final Study Report" by June 30, 2024.

Final Report Submission: June 30, 2024

8. Sarepta Therapeutics, Inc. commits to reassessing the commercial acceptance criterion for the release testing of potency of SRP-9001 drug product after data have been collected on (b) (4) commercial lots and submit a "Postmarketing Commitment – Final Study Report" by June 30, 2024.

   Final Report Submission: June 30, 2024

9. Sarepta Therapeutics, Inc. commits to implementing the following CMC change for the SRP-9001 (b) (4) The CMC change will be submitted as a "Postmarketing Commitment - Final Study Report" by December 31, 2024.

   Final Report Submission: December 31, 2024

10. Sarepta Therapeutics, Inc. commits to performing (b) (4). The final report will be submitted as a "Postmarketing Commitment – Final Study Report" by December 31, 2024.

    Final Report Submission: December 31, 2024

We request that you submit information concerning chemistry, manufacturing, and control postmarketing commitments and final reports to this BLA, STN BL 125781. Please refer to the sequential number for each commitment.

Please use the following designators to prominently label all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Commitment – Correspondence Status Update**
- **Postmarketing Commitment – Final Study Report**
- **Supplement contains Postmarketing Commitment Final Study Report**

For each postmarketing commitment not subject to the reporting requirements of 21 CFR 601.70, you may report the status to FDA as a **Postmarketing Study**

Commitment – Correspondence Status Update. The status report for each commitment should include:

- the sequential number for each study as shown in this letter;
- the submission number associated with this letter;
- describe what has been accomplished to fulfill the non-section 506B PMC; and,
- summarize any data collected or issues with fulfilling the non-section 506B PMC.

When you have fulfilled your commitment, submit your final report as **Postmarketing Commitment – Final Study Report** or **Supplement contains Postmarketing Commitment Final Study Report**.

POST APPROVAL FEEDBACK MEETING

New biological products qualify for a post approval feedback meeting. Such meetings are used to discuss the quality of the application and to evaluate the communication process during drug development and marketing application review. The purpose is to learn from successful aspects of the review process and to identify areas that could benefit from improvement. If you would like to have such a meeting with us, please contact the Regulatory Project Manager for this application.

Sincerely,

Melissa Mendoza, JD
Director
Office of Compliance
and Biologics Quality
Center for Biologics
Evaluation and Research

Peter Marks, MD, PhD
Director
Center for Biologics
Evaluation and Research

Exhibit E

U.S. FOOD & DRUG ADMINISTRATION

Our STN: BL 125781/34

SUPPLEMENT APPROVAL
PMR FULFILLED
SUPPLEMENT ACCELERATED APPROVAL
June 20, 2024

Sarepta Therapeutics, Inc.
Attention: Patrick O'Malley
215 First Street
Cambridge, MA 02142

Dear Patrick O'Malley:

We have approved your request received December 21, 2023, to supplement your Biologics License Application (BLA) submitted under section 351(a) of the Public Health Service Act (PHS Act) for delandistrogene moxeparvovec-rokl to expand the approved indication to individuals at least 4 years of age for the treatment of Duchenne muscular dystrophy (DMD) in patients who are ambulatory and have a confirmed mutation in the *DMD* gene.

We have also approved your request to supplement your BLA submitted under section 351(a) of the PHS Act for delandistrogene moxeparvovec-rokl to expand the approved indication to individuals at least 4 years of age for the treatment of DMD in patients who are non-ambulatory and have a confirmed mutation in the *DMD* gene, according to the regulations for accelerated approval, 21 CFR 601.41. The DMD indication in non-ambulatory patients is approved under accelerated approval based on expression of ELEVIDYS micro-dystrophin.

The review of this supplement was associated with the following National Clinical Trial (NCT) numbers: NCT05096221 and NCT04626674.

ACCELERATED APPROVAL REQUIREMENTS

Under accelerated approval regulations statutory provisions and we may grant marketing approval for a biological product on the basis of adequate and well-controlled clinical trials establishing that the biological product has an effect on a surrogate endpoint that is reasonably likely, based on epidemiologic, therapeutic, pathophysiologic, or other evidence, to predict clinical benefit or on the basis of an effect on a clinical endpoint other than survival or irreversible morbidity. This approval requires you to study the biological product further, to verify and describe its clinical benefit, where there is uncertainty as to the relation of the surrogate endpoint to clinical benefit, or of the observed clinical benefit to ultimate outcome.

Approval under these statutory provisions and regulations requires, among other things, that you conduct adequate and well-controlled clinical trials to verify and describe clinical benefit attributable to this product.

ACCELERATED APPROVAL REQUIRED STUDIES

We remind you of your postmarketing requirement (PMR) specified in your submission of June 11, 2024.

1. Conduct and submit the results of a randomized, controlled trial to verify and confirm the clinical benefit of delandistrogene moxeparvovec-rokl in patients with Duchenne's muscular dystrophy, who are non-ambulatory and have a confirmed mutation in the DMD gene. The trial should evaluate the effects of delandistrogene moxeparvovec-rokl on an endpoint that denotes clinical benefit.

   Final Protocol Submission: Submitted

   Study/Trial Completion: May 30, 2027

   Final Report Submission: November 30, 2027

We expect you to complete design, initiation, accrual, completion, and reporting of these studies within the framework described in your letter of June 11, 2024.

We acknowledge that you have provided the final protocol to your IND 17763. Please provide a letter of cross-reference to this BLA, STN BL 125781, explaining that this protocol was submitted to the IND. Please refer to the sequential number for each trial and the submission number as shown in this letter.

You must conduct this trial with due diligence. If required postmarketing trial(s) fail to verify that clinical benefit is conferred by delandistrogene moxeparvovec-rokl, or are not conducted with due diligence, including with respect to the conditions set forth below, we may withdraw this approval.

You must submit reports of the progress of each trial listed above as required under section 506(c) of the Federal Food, Drug, and Cosmetic Act (FDCA) to this BLA 180 days after the date of approval of this BLA and approximately every 180 days thereafter (see section 506B(a)(2) of the FDCA) (hereinafter "180-day reports").

You are required to submit two 180-day reports per year for each open study or clinical trial required under 506(c) of the FDCA. The initial report will be a standalone submission and the subsequent report will be combined with your application's annual status report required under section 506B(a)(1) of the FDCA and 21 CFR 601.70. The standalone 180-day report will be due 180 days after the date of approval. Submit the subsequent 180-day report with your application's annual status report. Submit both of

these 180-day reports each year until the final report for the corresponding study or clinical trial is submitted.

Your 180-day report must include the information listed in 21 CFR 601.70(b). FDA recommends that you use form FDA 3989 PMR/PMC Annual Status Report for Drugs and Biologics, to submit your 180-day reports. Form FDA 3989, along with instructions for completing this form, is available on the FDA Forms web page at https://www.fda.gov/about-fda/reports-manuals-forms/forms.

Your 180-day reports, including both the standalone 180-day report submitted 180 days after the date of approval and the 180-day report submitted with your annual status report, must be clearly designated as **180-Day AA PMR Progress Report.**

FDA will consider the submission of your annual status report under section 506B(a)(1) of the FDCA and 21 CFR 601.70, in addition to the submission of reports 180 days after the date of approval each year, to satisfy the periodic reporting requirement under section 506B(a)(2) of the FDCA. You are also required to submit information related to your confirmatory trial as part of your annual reporting requirement under section 506B(a)(1) of the FDCA until the FDA notifies you, in writing, that the Agency concurs that the study requirement has been fulfilled or that the study either is no longer feasible or would no longer provide useful information.

Label your annual report as an **Annual Status Report of Postmarketing Requirements/Commitments** and submit it to the FDA each year within 60 calendar days of the anniversary date of your original BLA until all Postmarketing Requirements and 506B Commitments are fulfilled or released.

Please submit the final study report as a supplement to this BLA, STN BL 125781. For administrative purposes, all submissions related to this postmarketing study requirement must be clearly designated as "**Subpart E Postmarketing Study Requirements**."

FULFILLED ACCELERATED APPROVAL REQUIRED STUDIES

We approved BLA STN BL 125781/0 on June 22, 2023, under 21 CFR 601 Subpart E for Accelerated Approval of Biological Products for Serious or Life-Threatening Illnesses. Approval of this supplement fulfills the following postmarketing requirement for an ongoing, randomized, double-blinded clinical trial intended to describe and verify clinical benefit of delandistrogene moxeparvovec-rokl in ambulatory patients with Duchenne muscular dystrophy (DMD) made under 21 CFR 601.41.

STN: BL 125781/0

PMR #1: Complete Study SRP-9001-301 Part 1, an ongoing, randomized, double-blinded clinical trial intended to describe and verify clinical benefit of delandistrogene moxeparvovec-rokl in ambulatory patients with Duchenne muscular dystrophy (DMD). The trial evaluates the primary endpoint of

North Star Ambulatory Assessment (NSAA) and compares delandistrogene moxeparvovec-rokl to placebo in 125 ambulatory patients with DMD with confirmed mutation in the DMD gene.

Final Protocol Submission: September 8, 2021

Study/Trial Completion: September 13, 2023

Final Report Submission: January 11, 2024

LABELING

We hereby approve the draft content of labeling Package Insert submitted under amendment 21, dated June 17, 2024.

WAIVER OF HIGHLIGHTS

We are waiving the requirements of 21 CFR 201.57(d)(8) regarding the length of Highlights of prescribing information. This waiver applies to all future supplements containing revised labeling unless we notify you otherwise.

CONTENT OF LABELING

As soon as possible, but no later than 14 days from the date of this letter, please submit the final content of labeling (21 CFR 601.14) in Structured Product Labeling (SPL) format via the FDA automated drug registration and listing system, (eLIST) as described at http://www.fda.gov/ForIndustry/DataStandards/StructuredProductLabeling/default.htm. Content of labeling must be identical to the Package Insert submitted on June 17, 2024. Information on submitting SPL files using eLIST may be found in the guidance for industry *SPL Standard for Content of Labeling Technical Qs and As* at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072392.pdf.

The SPL will be accessible via publicly available labeling repositories.

All final labeling should be submitted as Product Correspondence to this BLA, STN BL 125781 at the time of use and include implementation information on Form FDA 356h.

ADVERTISING AND PROMOTIONAL LABELING

You may submit two draft copies of the proposed introductory advertising and promotional labeling with Form FDA 2253 to the Advertising and Promotional Labeling Branch at the following address:

Food and Drug Administration
Center for Biologics Evaluation and Research
Document Control Center
10903 New Hampshire Ave.
WO71–G112
Silver Spring, MD 20993-0002

You must submit copies of your final advertising and promotional labeling at the time of initial dissemination or publication, accompanied by Form FDA 2253 (21 CFR 601.12(f)(4)).

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence or substantial clinical experience to support such claims (21 CFR 202.1(e)(6)).

For each pending supplemental application for this BLA that includes proposed revised labeling, please submit an amendment to update the proposed revised labeling with the changes approved today.

PROMOTIONAL MATERIALS

Please note that the accelerated approval regulation concerning promotional materials (21 CFR 601.45) stipulates that all advertising and promotional labeling items that you wish to distribute in the first 120 days following approval must have been received by FDA prior to the approval date. After approval, promotional items intended for dissemination after the first 120 days following approval must be submitted to the FDA at least 30 days prior to the anticipated distribution date. Please submit draft materials with a cover letter noting that the items are for accelerated approval and an accompanying FORM FDA 2253 to the Advertising and Promotional Labeling Branch at the following address:

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10903 New Hampshire Ave.
WO71-G112
Silver Spring, MD 20993-0002

You must submit copies of your final advertisement and promotional labeling at the time of initial dissemination or publication accompanied by FORM FDA 2253 (21 CFR 601.12(f)(4)).

Alternatively, you may submit promotional materials for accelerated approval products electronically in eCTD format. For more information about submitting promotional materials in eCTD format, see the draft guidance for industry available at

http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM443702.pdf.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence or substantial clinical experience to support such claims (21 CFR 202.1(e)(6)).

PEDIATRIC REQUIREMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because the biological product for this indication has an orphan drug designation, you are exempt from this requirement.

We will include information contained in the above-referenced supplement in your BLA file.

Sincerely,

Peter Marks, MD, PhD
Director
Center for Biologics Evaluation and Research

Exhibit F

U.S. FOOD & DRUG ADMINISTRATION

CBER/DMPQ CMC/Facility BLA Review Memorandum

BLA STN 125781/0

delandistrogene moxeparvovec (SRP-9001)

Ou Olivia Ma, DMPQ
Reviewer

DMPQ review memo by Ou Olivia Ma

Sarepta BLA 125781/0

1. BLA#: STN 125781/0

2. APPPLICANT NAME AND LICENSE NUMBER

Name: Sarepta Therapeutics, Inc.

US License #: 2308

3. PRODUCT NAME/PRODUCT TYPE

Proper name: **delandistrogene moxeparvovec (SRP-9001)**

Proprietary name: **N/A**

4. GENERAL DESCRIPTION OF THE FINAL PRODUCT

a. Pharmacological category: **Gene Therapy**

b. Dosage form: **Solution for Infusion**

c. Strength/Potency: **1.33E+13 vector genomes (vg)/mL**

d. Route of administration: **Intravenous infusion**

e. Indication(s): **Treatment of ambulatory patients with Duchenne muscular dystrophy (DMD) with a confirmed mutation in the DMD gene**

5. MAJOR MILESTONES

- Application Receipt Date: September 28, 2022
- First Committee Meeting: October 24, 2022
- Filing Meeting: November 12, 2022
- Filing Action: November 27, 2022
- Internal Mid-Cycle Meeting: January 12, 2023
- Mid-Cycle Communication: January 24, 2023
- Late-Cycle Meeting: March 14, 2023
- Advisory Committee Meeting: May 12, 2023
- PDUFA Action Due Date: May 29, 2023
- Pre-License Inspection (PLI) of Catalent Biopark facility: February 21-24, 2023
- PLI of Catalent BWI facility: March 6-10, 2203
- PLI of Sarepta testing facility in Andover, MA: March 20-24, 2023

6. DMPQ CMC/FACILITY REVIEW TEAM

Reviewer/Affiliation	Section/Subject Matter
Ou Olivia Ma, OCBQ/DMPQ/MRB2	Drug substance, Drug Product, Facilities

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7. SUBMISSION(S) REVIEWED

Date Received	Submission	Comments/ Status
Sep 28, 2022	STN 125781/0	
Jan 12, 2023	Amendment STN 125781/0/13 (Response to IR sent on Dec 27, 2022)	Reviewed and found acceptable
May 19, 2023	Amendment STN 125781/0/61 (Response to Catalent BWI inspection Form FDA 483)	Reviewed and found acceptable

8. REFERENCED REGULATORY SUBMISSIONS (e.g., IND BLA, 510K, Master File, etc.)

Submission Type & #	Holder	Referenced Item	Letter of Cross-Reference	Comments/Status
DMF (b) (4)	(b) (4)	(b) (4) vials	yes	No DMF review required, information pertinent to container closure is provided in the BLA
DMF (b) (4)	(b) (4)	(b) (4) Vials	yes	No DMF review required, information pertinent to container closure is provided in the BLA
DMF (b) (4)	(b) (4)	Stopper/(b) (4)	yes	No DMF review required, information pertinent to container closure is provided in the BLA
STN (b) (4)	(b) (4)	Stopper, elastomeric formulations, coatings, films	yes	Deferred to OTP reviewers

9. REVIEWER SUMMARY AND RECOMMENDATION

A. EXECUTIVE SUMMARY

Sarepta Therapeutics, Inc. (Sarepta) submitted documentation to BLA STN 125781/0 to support licensure of delandistrogene moxeparvovec (SRP-9001), a gene therapy product intended to treat ambulatory patients with Duchenne muscular dystrophy (DMD) with a

confirmed mutation in the DMD gene. CBER/DMPQ reviewed and evaluated the drug substance (DS) and drug product (DP) manufacturing processes and facilities proposed for use in the manufacture of SRP-9001. Information reviewed, evaluated, and documented in this memo includes data to validate and support the consistency of the manufacturing process and product quality; facility information which includes utilities, cross-contamination prevention measures, and maintenance of controlled environments; and equipment for use in the manufacturing (all product-contact equipment used in DS and DP manufacturing are single-use).

As part of the BLA review, three Pre- License Inspections (PLIs) were performed including the PLI of the DP manufacturing facility at Catalent Biopark in Baltimore, MD on February 21-24, 2023, DS manufacturing facility at Catalent BWI in Harmans, MD on March 6-10, 2023, and a DP release testing facility at Sarepta in Hanover, MA on March 20-24, 2023. Each PLI was documented in a separate establishment inspection report (EIR).

At the conclusion of the Catalent BWI inspection, a Form FDA 483 was issued on March 10, 2023 with two inspectional observations, to which the firm responded on March 31, 2023. At the conclusion of the Sarepta facility inspection, a Form FDA 483 was issued on March 24, 2023 with one inspectional observation, to which the firm responded on April 12, 2023. All inspectional 483 observations were deemed resolved, and both the Catalent BWI and Sarepta facilities were classified as Voluntary Action Indicated (VAI). No Form 483 was issued at the conclusion of the Catalent Biopark PLI, and this PLI was classified as No Action Indicated (NAI).

In addition to the PLIs, facility inspections were waived for the DP packaging and labeling facility of (b) (4), and the DP release testing facilities of (b) (4) in (b) (4). The inspection waivers were based on the evaluations of the facilities' inspection compliance histories. The inspection waivers are documented in a separate inspection waiver memo dated February 10, 2023.

This submission was granted priority review with 8-month review cycle.

B. RECOMMENDATION

I. APPROVAL

Based on the review of the information submitted to BLA 125781/0 and in conjunction with the PLIs and inspectional compliance history evaluations, the production process, facilities, equipment, and controls appear acceptable; approval is recommended with the following inspectional recommendation. CBER understands that the recommendation may or may not be taken (based on risk and available resources) and is not requesting documentation to be submitted as evidence of completion.

- On the next inspection of Catalent Maryland (BWI), (b) (5), (b) (7)(E)

Below is a listing of the Drug Substance (DS) and Drug Product facilities to be included in the approval letter:

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- DS manufacturing facility:

  Catalent Pharma Services
  Catalent Maryland (BWI)
  7555 Harmans Road
  Harmans, MD 20177, USA
  FEI#: 3015434301
  DUNS#: 116950534

- DP manufacturing facility:

  Catalent Pharma Solutions
  Catalent Maryland (BioPark)
  801 West Baltimore Street, Suite 302
  Baltimore, MD 21201, USA
  FEI#: 3015558590
  DUN#: 618890289

Below is the list of approvable Comparability Protocol(s) (CP):

- CP for conducting additional drug substance batches in manufacturing Suite (b) (4) at the Catalent BWI facility.

II. SIGNATURE BLOCK

Reviewer/Title/Affiliation	Concurrence	Signature and Date
Ou (Olivia) Ma/ Consumer Safety Officer OCBQ/DMPQ/MRB2	Concur	
Anthony Lorenzo / Branch Chief OCBQ/DMPQ/MRB2	Concur	
Carolyn Renshaw / Division Director OCBQ/DMPQ	Concur	

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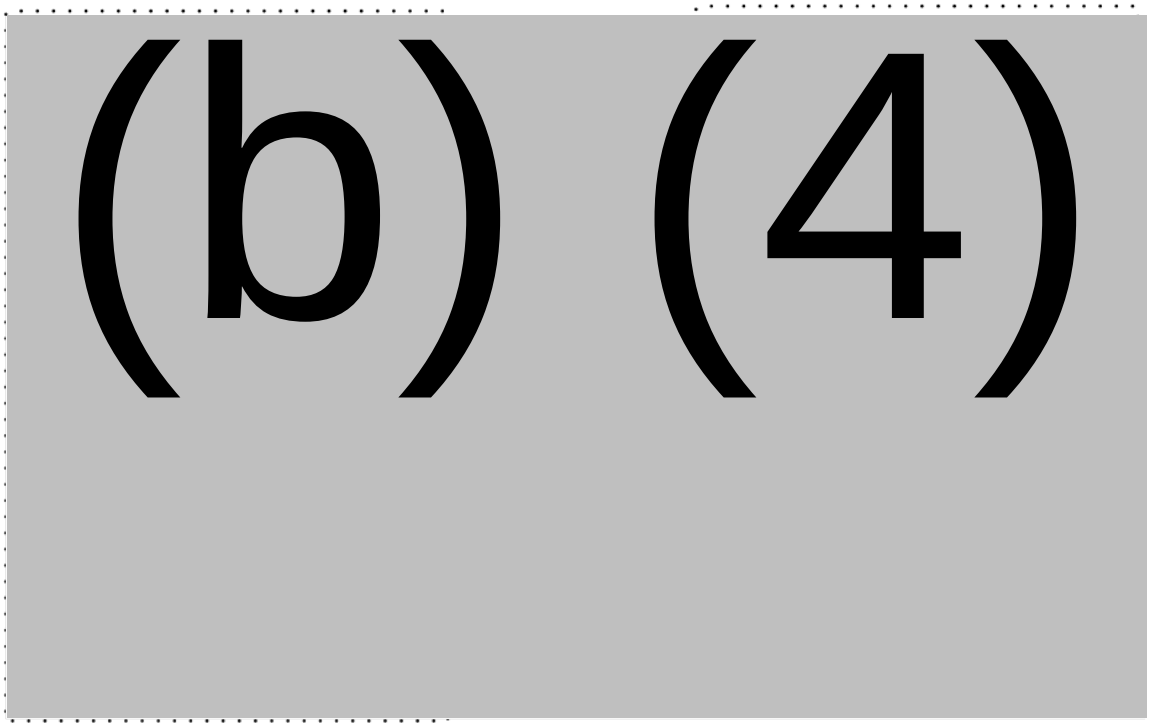
3.2.S DRUG SUBSTANCE

(b) (4)

3.2.S.2 Manufacture

3.2.S.2.1 Manufacturer(s)

(b) (4)



10 pages determined to be not releasable: (b)(4)

(b) (4)

3.2.P DRUG PRODUCT

3.2.P.1 Description and Composition of the Drug Product

SRP-9001 1.33 X 10^{13} vg/mL solution for infusion (drug product) is supplied as a single-use, preservative-free, sterile, aqueous solution for intravenous infusion.

One vial contains 10 mL of 1.33 X 10^{13} vg/mL of Delandistrogene moxeparvovec (SRP-9001) formulated in a buffered solution of 20 mM Tromethamine/Tromethamine-HCl, 1 mM Magnesium chloride, 200 mM Sodium chloride, and 0.001% Poloxamer 188. Each vial contains an extractable volume of not less than 10.0 mL. The container closure system consists of a cyclic olefin polymer vial closed with a rubber stopper and sealed with an aluminum seal and plastic flip-off cap.

3.2.P.2.4 Container Closure System

Refer to section 3.2.P.7 Container Closure System for the primary container closure system description, specifications, and its qualification (per DMPQ purview).

3.2.P.2.5 Microbiological Attributes

The SRP-9001 drug product is manufactured as a sterile DP by aseptic processing, and supplied as preservative-free, single-use vials. The DS is manufactured using a (b) (4) to the DP(b) (4) process. As part of the aseptic filling process, DP solution is filtered through (b) (4). DP is aseptically filled using a validated process, and all product-contact components are either received sterile or sterilized during validated process. The container closure integrity of the primary packaging systems was demonstrated by (b) (4) testing with a detection limit of (b) (4). DP is subject to sterility and endotoxin testing as part of the release process, with acceptance criteria of no growth and (b) (4), respectively. Assurance of container closure system integrity during shipping was established by the shipping validation study.

Reviewer's Assessment: *The microbial attributes and control strategy appears acceptable. The*(b) (4) *controlled DS manufacturing process is reviewed in Section 3.2.S.2.4. The sterile filtration steps are reviewed in Sections 3.2.P.3.3 and 3.2.P.3.4. DP filling aseptic process validation, as well as product-contact material sterilization process validation are reviewed in Section 3.2.P.3.5. Container closure integrity testing validation is reviewed in Sections 3.2.P.5.3 and 3.2.P.7.*

3.2.P.3 Manufacture

3.2.P.3.1 Manufacturer(s)

Refer to section 3.2.A.1 for a complete list of DP manufacturing facilities.

3.2.P.3.3 Description of Manufacturing Process

The SRP-9001 DP manufacturing process is performed at Catalent Maryland (Biopark) facility in Baltimore, MD, and include the following steps.

Formulation buffer preparation: formulation buffer is prepared by (b) (4) The final concentration of the formulation buffer is 20 mM Tris, 200 mM sodium chloride, 1 mM magnesium chloride, 0.001% poloxamer 188, and (b) (4) The formulation buffer is tested for(b) (4)

(b) (4)

Sterile filtration: The formulated bulk drug product solution is sterile (b) (4)

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(b) (4)

Aseptic filling, stoppering, and capping: Aseptic filling, stoppering, and capping is performed on (b) (4) Each vial is filled to the target fill volume and fill (b) (4) checks are manually performed at predefined intervals throughout the fill.

Visual inspection: All filled vials are manually inspected for container closure and solution defects within (b) (4). Vials passing the 100% manual visual inspection process are then sampled for an Acceptable Quality Limit (AQL) visual inspection.

Freeze and storage: The drug product vials are frozen and stored at ≤ -60°C.

Labeling and packaging: Drug product is transferred to the secondary packaging site. Labeling and packaging operations are performed at (b) (4)

Long-term storage: The final drug product vials are stored at ≤ -60°C.

Drug product batches produced at Catalent Biopark are numbered using the following nomenclature: A-634-SRP9001-20-XXXX, with A: Drug Product; 634: designation of company (Sarepta); SRP9001: designation of drug product; 20: (b) (4) XXXX: (b) (4)

Reviewer's Assessment: *Adequate information is provided for the DP process description. Description and assessment of controls associated with critical steps operating and performance parameters, in-process controls and hold-times are provided in sections 3.2.P.3.4 Controls of Critical Steps and Intermediates, and 3.2.P.3.5 Process Validation.*

3.2.P.3.4 Controls of Critical Steps and Intermediates

The formulation buffer is (b) (4)

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Reviewer's Assessment: *Microbial control strategy including sterility assurance steps and in-process control testing appears suitable. The bioburden testing* (b) (4) *is in place to ensure contamination control of the drug product* (b) (4) *processing and reduce the risk of sterility failure.* (b) (4) *integrity test is in place to ensure no microbial breach of the sterilizing filter.*

Maximum in-process hold times for each process step have been identified. Review of the microbial qualities in support of the maximum hold time for the formulation buffer is reviewed in Section 3.2.P.3.5. Evaluation of all other maximum allowable hold time studies is deferred to OTP reviewers. Validation of aseptic filling is reviewed in section 3.2.P.3.5.

3.2.P.3.5 Process Validation and/or Evaluation

(b) (4)

(b) (4)

(b) (4)

1 page determined to be not releasable: (b)(4)

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3.2.P.5 Control of Drug Product

3.2.P.5.1 and 3.2.P.5.6 Specification(s) and Justification of Specification(s)

DP specifications under DMPQ purview are reviewed in section 3.2.P.2.5 Microbiology Attributes.

3.2.P.5.2 and 3.2.P.5.3 Analytical Procedures and Validation of Analytical Procedures

CCIT and its method validation is reviewed in Section 3.2.P.7 Container Closure System. Evaluation of other analytical methods is deferred to OTP and DBSQC reviewers.

3.2.P.5.4 Batch Analyses

Batch analyses results are provided for (b) (4) lots that were manufactured from (b) (4) with the commercial process. These batches cover nonclinical lots, clinical lots, engineering runs, and DS and DP process validation batches including the three consecutive DP PPQ lots (b) (4) Batch sizes range from (b) (4) vials.

Under DMPQ purview, the batch analyses testing including sterility and endotoxin tests and the acceptance criteria are no growth and (b) (4), respectively. All batches met the sterility acceptance criteria, and all batches met the endotoxin acceptance criteria with the highest endotoxin level at (b) (4)

Reviewer's Assessment*: Lots included in the batch analyses appear suitable, including the three consecutive DP validation runs. There were no deviations for the batch testing under DMPQ purview. Additional data for batches manufactured by an archived process ("Process A") were also provided. As this process is no longer being used for SRP-9001 DP manufacturing, the analyses are not included in this review.*

3.2.P.7 Container Closure System

Components of the Container Closure System

The container closure system consists of cyclic olefin polymer (COP) vials, stoppered with grey rubber stoppers, and further sealed with aluminum seals with flip-off plastic caps. The components of the container closure system are listed in Table 5:

Table 5. SRP-9001 DP Container Closure Components

Component	Description	Manufacturer	Standards
Vial	10-mL, cyclic olefin polymer (b) (4)	(b) (4)	(b) (4)

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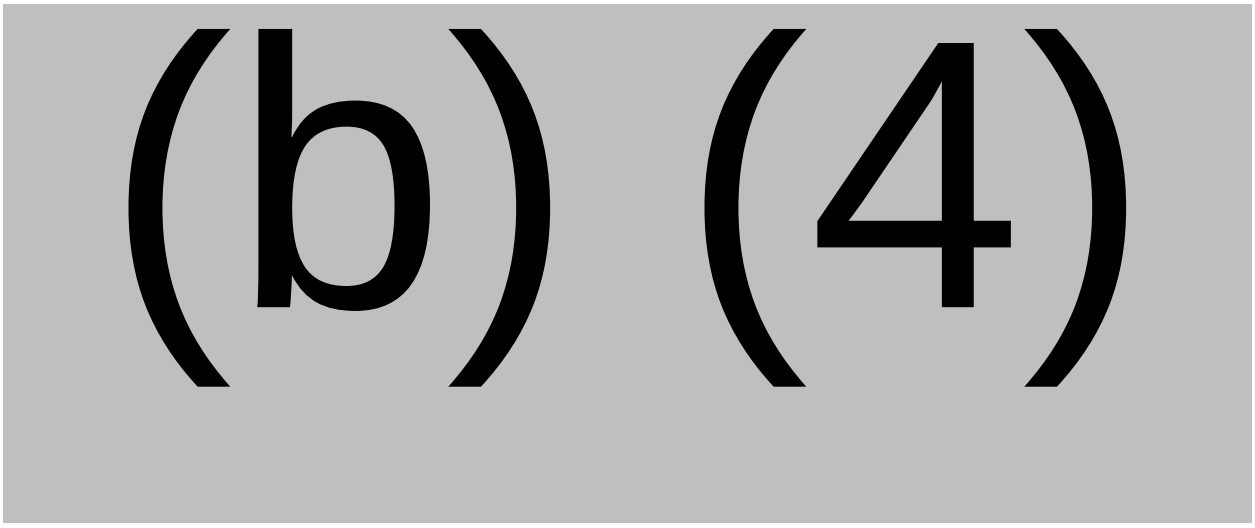
Component	Description	Manufacturer	Standards
Stopper	20-mm, (b) (4) grey chlorobutyl rubber stopper with (b) (4) barrier on product contact side, (b) (4) coating on non-product side	(b) (4)	(b) (4)
Cap	20-mm, aluminum shell with polypropylene flip- off cap overseal	(b) (4)	Not applicable (no product contact)

All components in the SRP-9001 DP primary packaging system are received sterile and ready to use. The specifications for the vials, stoppers and caps are summarized in Table 6. The components of the container closure system are released against these specifications based on the Certificate of Analysis (CoA) provided by the manufacturer.

Table 6. SRP-9001 DP Container Closure Specifications



The selected container closure system (CCS) for the drug product is commonly used in the industry for cell and gene therapies due to its performance properties of break resistance and structural integrity under cryogenic storage conditions. The cyclic olefin polymer (COP) (b) (4) are chosen for the primary container closure since SRP-9001 vials are stored at ≤ -60°C and polymer vials are more robust and supportive than glass at low temperature. Rubber stoppers and COP polymer have more similar coefficients (than glass) of thermal expansion, reducing the risk of ingress.

The secondary packaging consists of an opaque, tamper-evident, rigid paperboard carton. The carton provides physical protection for the vials during storage and shipping.

CCIT by (b) (4) Testing

CCIT by (b) (4) testing is performed to define the crimping settings and is part of the stability testing program. (b) (4) testing is performed by (b) (4) and the test

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method has been validated for the SRP-9001 container closure system per (b) (4)

(b) (4)

Reviewer's Assessment: *It appears acceptable to use* (b) (4) *solution filled vials as surrogate for the SRP-9001 drug product. The* (b) (4) *limit and quantitation limit of the* (b) (4) *test for the SRP-9001 drug product container closure appear to be appropriately validated.*

(b) (4)

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(b) (4)

3.2.P.8 Stability

3.2.P.8.1 Stability Summary and Conclusion and 3.2.P.8.3 Stability Data

The stability studies include storage for up to (b) (4) months at the long-term condition of ≤ -60°C or at accelerated condition of (b) (4) The proposed shelf life is 12 months stored at ≤ -60°C.

(b) (4) batches of drug product are being evaluated on stability, including (b) (4) primary stability lots (registration stability batches) and (b) (4) process validation batches. The vials are stored in only one configuration (upright) on stability (b) (4)

For both storage conditions, container closure integrity testing is scheduled for 12, (b) (4) (b) (4) (b) (4) sterility is tested at study start with an acceptance criterion of no growth, and endotoxin testing is scheduled for 0, 6, 12, (b) (4) months with an acceptance criterion of (b) (4) . For the (b) (4) storage condition, additional CCIT was performed at (b) (4) months.

Stability data of up to 12 months storage at both conditions were provided and sterility, endotoxin and CCIT testing all conform to acceptance criteria.

Reviewer's Assessment: *The sterility, endotoxin and CCIT testing schedules on the stability study appear acceptable. The testing results support the shelf life of 12 months stored at ≤ -60°C from DMPQ perspective.*

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3.2.A APPENDICES

Facilities Table

Manufacturing/ Testing activities	Inspection ? Waiver? or Not Required?	Compliance Check Required for Approval?	RMS-BLA Entry Required ?	Comments
Facility: Catalent Pharma Services Catalent Maryland (BWI) 7555 Harmans Road Harmans, MD 20177, USA **FEI#:** 3015434301 **DUNS#:** 116950534 DS Manufacturing; DS In-process, Release and Stability Testing, DS Labeling and Storage; Master Cell Bank Storage; Working Cell Bank Storage	Inspection	Yes	Yes	Pre-license Inspection for STN 125781/0, VAI, Mar 6-10, 2023
Facility: Catalent Pharma Solutions Catalent Maryland (BioPark) 801 West Baltimore Street, Suite 302 Baltimore, MD 21201, USA **FEI#:** 3015558590 **DUN#:** 618890289 DP manufacturing; Working Cell Bank Manufacturing, Testing and Storage; Master Cell Bank Storage	Inspection	Yes	Yes	Pre-license Inspection for STN 125781/0, NAI, Feb 21-24, 2023
Facility: (b) (4) Labeling; Secondary packaging; DP storage	Waiver	Yes	Yes	Surveillance inspection, NAI, (b) (4)

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Manufacturing/ Testing activities	Inspection ? Waiver? or Not Required?	Compliance Check Required for Approval?	RMS-BLA Entry Required ?	Comments
Facility: Sarepta Therapeutics 100 Federal Street Andover, MA 01810, USA **FEI#:** 3012807588 **DUNS#:** 072827382 DS Release and Stability Testing; DP Release and Stability Testing	Inspection	Yes	Yes	Pre-license Inspection for STN 125781/0, VAI, Mar 20-24, 2023
Facility: (b) (4) DS In-Process and Release Testing; DP Release and Stability Testing	Waiver	Yes	Yes	Surveillance inspection, VAI, (b) (4)
Facility: (b) (4) DP Release Testing	Waiver	Yes	Yes	Surveillance inspection, VAI, (b) (4)
Facility: (b) (4) Master Cell Bank and Working Cell Bank Manufacturing and Testing	Not required	No	Yes	Site has been decommissioned and no further activities will occur here.

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Manufacturing/ Testing activities	Inspection ? Waiver? or Not Required?	Compliance Check Required for Approval?	RMS-BLA Entry Required ?	Comments
Facility: (b) (4) DS Release and Stability Testing	Not required	No	Yes	
Facility: (b) (4) Master Cell Bank and End of Production Cell Bank Testing	Not required	No	Yes	
Facility: (b) (4) Working Cell Bank and End of Production Cell Bank Testing; DS Release Testing	Not required	No	Yes	
Facility: (b) (4) Master Cell Bank, Working Cell Bank and End of Production Cell Bank Testing; DS Release Testing	Not required	No	Yes	

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Manufacturing/ Testing activities	Inspection ? Waiver? or Not Required?	Compliance Check Required for Approval?	RMS-BLA Entry Required ?	Comments
Facility: (b) (4) Master Cell Bank and End of Production Cell Bank Testing	Not required	No	Yes	
Facility: (b) (4) DS In-process Testing	Not required	No	Yes	
Facility: (b) (4) DS Release Testing; (b) (4) Cell Bank and (b) (4) Release Testing	Not required	No	Yes	
Facility: (b) (4) (b) (4) Cell Bank and (b) (4) Manufacturing, Testing and Storage	Not required	No	No	Surveillance inspection, NAI, (b) (4)

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Manufacturing/ Testing activities	Inspection ? Waiver? or Not Required?	Compliance Check Required for Approval?	RMS-BLA Entry Required ?	Comments
Facility: (b) (4) (b) (4) Release Testing	Not required	No	No	
Facility: (b) (4) (b) (4) Release Testing	Not required	No	No	
Facility: (b) (4) (b) (4) Cell Bank Release Testing	Not required	No	No	
Facility: (b) (4) (b) (4) Cell Bank and (b) (4) Lot Storage	Not required	No	No	

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Manufacturing/ Testing activities	Inspection ? Waiver? or Not Required?	Compliance Check Required for Approval?	RMS-BLA Entry Required ?	Comments
Facility: (b) (4) (b) (4) Cell Bank and (b) (4) Lot Storage	Not required	No	No	
Facility: (b) (4) DP Stability Testing	Not required	No	No	Container closure integrity test for stability

Catalent BWI DS Manufacturing Facility

Facility Design

SRP-9001 drug substance is manufactured at Catalent BWI facility located in Harmans, Maryland. Catalent BWI is a multi-product manufacturing facility for commercial and clinical manufacturing of gene therapy products. The following product types are manufactured at Catalent BWI facility:

- AAV vectors for gene therapy (adeno-associated virus vectors)
- AAV vectors for gene editing/cell therapy (CAR-T cell)
- Non-replicating recombinant adeno-associated virus
- Master Cell Banks (HEK293, Lentiviral, (b) (4)

Catalent BWI facility consists of (b) (4)

7 pages determined to be not releasable: (b)(4)

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(b) (4)

DS Shipping Validation

(b) (4)

Catalent Biopark DP Manufacturing Facility

Facility Design

SRP-9001 drug product is manufactured at Catalent Biopark located on the University of Maryland, Baltimore campus. The building is approximately (b) (4) square feet, and consists of corporate office, general laboratory, and GMP manufacturing areas including areas for formulation, filling, visual inspection, and cell banking. Catalent Biopark is a multi-product facility specializing in the commercial and clinical manufacturing operations of gene therapy products including adeno-associated viral vector, recombinant protein, and oncolytic adenoviral vector. The site also manufactures working cell banks (293T cells) and has the capacity of producing recombinant protein using mammalian cells. Among these products, SRP-9001 is the first product seeking commercial approval.

For SRP-9001 production, DP aseptic filling, stoppering and capping take place in Room (b) (4) which is a Grade (b) (4) within a Grade (b) (4) space (Room (b) (4) DP formulation and sterile filtration take pace in the (b) (4) in Grade (b) (4) Room (b) (4) DP formulation buffer is prepared in Room (b) (4) (Grade (b) (4)

All Grade (b) (4) manufacturing space is accessed through Grade (b) (4) material air lock (MAL) and personnel air lock (PAL), which then connect to the Grade (b) (4) corridor. Material transfer requires (b) (4) as containers of materials move through the facility.

The facility enforces strict training and gowning requirements for the manufacturing areas. Entry into the Grade (b) (4) manufacturing hallway is gained by passing through a locker room and putting on appropriate gowning. Access to the Grade (b) (4) manufacturing

area is through a (b) (4) airlock configuration. Passage between the classified hallway and formulation suites, equipment wash/storage/prep areas is through additional airlocks to minimize cross contamination and maintain overall cleanliness.

Where specifications allow, all equipment and components are prepared for aseptic processes using validated autoclave cycles in a qualified, (b) (4) -supplied autoclave. All other equipment, media, and components are supplied in a qualified, aseptic, or sterile condition by appropriate vendors or prepared under an approved compliant aseptic procedure.

All controlled, classified areas are cleaned and sanitized on a regular basis per a Standard Operating Procedure. Standard Operating Procedures describe the frequency and type of cleaning and sanitization to be performed for each production area. Sanitizing agents are qualified for effectiveness on clean room surfaces using representative micro-organisms, including selected environmental isolates. House environmental isolates are selected based on the environmental program from which data is reviewed on regularly.

Prevention of contamination and cross-contamination

Prevention of contamination and cross-contamination is ensured through engineering, procedural, and manufacturing controls.

The facility design is the primary infrastructure supporting contamination control. Exposed surfaces in classified areas are constructed with smooth, non-porous materials to minimize contamination and withstand repeated cleaning. Flat surfaces and recesses are minimized to reduce the potential for contaminate accumulations; false ceilings are sealed; and sinks and drains are not within Grade (b) (4) areas.

A dedicated Air Handling Units (AHUs) controls the manufacturing areas on the (b) (4) floor, with HEPA filtration for (b) (4) . Pressure differentials and airlocks are set up to ensure area classifications and Biosafety Level (BSL) qualification. Primary containment to prevent contamination includes using (b) (4)

Procedural controls are established for material, personnel, and waste flows including gowning requirements, use of airlocks, spillage handling, and restricted key card access. All equipment that has direct product contact for SRP-9001 manufacture is single use.

Activities to clean and inactivate possible virus residues include (b) (4) All cleaning, decontamination and sterilization processes and reagents are qualified. Cleaning and changeover procedures are performed between products campaigns.

Reviewer's Comment*: The facility containment features, and cross-contamination control procedures appear acceptable. The cleaning and changeover procedures were evaluated during the PLI of February 21-24, 2023.* (b) (4) *cleaning and decontamination is defined by approved SOP. After each filling and prior to product changeover,* (b) (4)

surfaces are decontaminated by ready-to-use (b) (4) *with sterile* (b) (4) *after a* (b) (4) *contact time.* (b) (4) *decontamination is documented in the filling machine usage logbook. No issues were noted; procedures appeared acceptable.*

Facility cleaning and disinfectant effectiveness studies

Reviewer's Comment*: Facility cleaning and disinfectant effectiveness were reviewed during the PLI of February 21-24, 2023 and found acceptable. Refer to Establishment Inspection Report for details.*

Critical Utilities

Water

Water For Injection (WFI) used for manufacturing is sourced from qualified vendors and is not produced on site. Purified Water (PW) systems are not utilized for the manufacturing process but for the (b) (4) (e.g., (b) (4)). The PW system is qualified and meets (b) (4) for (b) (4)

Reviewer's Comment*: The WFI used in manufacturing is not produced on site. The qualification and monitoring of* (b) (4) *-PW appears acceptable. Water system was evaluated in detail during the PLI of February 21-24, 2023; no issues were noted.*

HVAC

The Heating, Ventilation and Air Conditioning (HVAC) systems that support the cleanrooms are designed to purify and condition the air supplied to the suites through filtration, predefined air changes per hour and control of temperature, relative humidity, and differential pressures. The AHUs provide the cleanrooms with (b) (4) HEPA-filtered air to achieve the appropriate temperature, humidity, airflow, and positive pressure to meet the (b) (4) classification for each cleanroom. HEPA filters are rated 99.99% efficient and HEPA certification is performed per (b) (4) standards.

AHU(b) (4) controls the entire SRP-9001 manufacturing areas, including the Grade(b) (4) and Grade(b) (4) manufacturing areas, Grade (b) (4) airlocks, and Grade(b) (4) hallways, preparation suites and gowning rooms.

Cleanroom temperatures are maintained between (b) (4) while relative humidity (RH) is maintained between (b) (4) . Cleanrooms and airlocks are controlled with an interlocking system to maintain positive pressure differentials and minimize reverse airflow.

The room pressurization conceptual design is for all cleanrooms to be positive to the external environment. Differential Pressures are maintained between adjacent areas within the suite based on the intended work being performed (upstream, downstream, and fill/finish) in the area. Room pressurization is controlled to maintain the minimum differential pressure levels to provide the level of contamination control as appropriate. DP levels and alarms are continuously monitored by the (b) (4) system.

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HVAC and HEPA filters are requalified (b) (4) . The (b) (4) (Fill Finish Suite) GMP HVAC was last qualified over a (b) (4) period from (b) (4) .

Reviewer's Comment: *The HVAC zoning and pressure differential control appear acceptable. The requalification of the HVAC was reviewed in detail during the PLI of February 21-24, 2023. Refer to the Establishment Inspection Report for details.*

Please note that in this BLA submission, Sarepta stated that the HVAC and HEPA are requalified (b) (4) at Catalent Biopark. Per "Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing – Current Good Manufacturing Practice", HEPA filter leak test shall be performed twice a year for Grade A and Grade B manufacturing areas. This requirement was conveyed to Catalent Biopark during the February 21-24, 2023 inspection, and Catalent Biopark updated the HVAC requalification SOP promptly to reflect this change.

Computer Systems

The automatic filler is controlled by HMI Panel. Filling settings (e.g., line speed) are entered before each filling, and no electronic data are generated during filling.

The software systems used at Catalent Biopark facility are the same as the ones used at Catalent BWI facility, except that (b) (4) Process Control System is not used at Catalent Biopark. Refer to the Computer Systems section under Catalent BWI facility for details.

Reviewer's Comment: *A general description of the computer systems used at the Catalent Biopark facility was provided and reviewed. The computer systems appear acceptable.*

Equipment

After formulation, (b) (4)

Major reusable equipment used in the SRP-9001 DP manufacturing process is summarized in Table 14:

Table 14. List of SRP-9001 DP Manufacturing Equipment

Equipment	Product Contact	Product Dedicated
Freezer	No	No
(b) (4)	No	No
(b) (4)	No	No
(b) (4)	No	No

(b) (4) filling machine	No	No

All reusable equipment was determined to not be in direct contact with product, and the non-product contact equipment is cleaned according to established procedures. Major process equipment utilized in drug product manufacturing has been qualified. For the (b) (4) filling machine, filling volume performance qualification was completed to verify the fill accuracy and capability.

In addition to these cleaning procedures, a (b) (4) is completed after viral agent processing. The (b) (4) includes a (b) (4) . Decontamination verification is achieved through (b) (4)

A risk assessment was performed to identify the risks associated with cleaning verification/validation of equipment used in the manufacturing of SRP-9001 drug product. Based on the outcome of the assessment, it was determined that cleaning verification/validation was not required.

Note that the SRP-9001 filling process does not use an (b) (4) but instead, uses a (b) (4) the filling line. Contamination control and sterility assurance for SRP-9001 drug product is achieved through the use of onsite- or pre-sterilized, single-use processing components for filtration and filling operations.

Reviewer's Comments: *Equipment used in the SRP-9001 DP manufacture is not product contact. Equipment qualification is reviewed during the PLI of February 21-24, 2023; no issues were noted.* (b) (4) *qualification is reviewed in the Sterilization section below.*

Sterilization

Filling (b) (4) Sterilization by (b) (4)

The filling (b) (4) is the only material that has product-contact after the sterilizing filtration step. The filling (b) (4) is single-use and received sterilized and ready-to-use. Sterilization is performed at (b) (4)

(b) (4)

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(b) (4)

Reviewer's Assessment: *The validation of the* (b) (4) *of the filling* (b) (4) *includes dose map study, worst-case evaluation,* (b) (4) *monitoring,* (b) (4) *, and low verification dose study. The study design and results appear acceptable.*

Filter Sterilization by (b) (4)

(b) (4)

After initial qualification, the equipment and sterilization parameters are revalidated (b) (4) . The last requalification for (b) (4) are Mar 07, 2022 and Apr 07, 2022, respectively, and all studies met the predefined acceptance criteria.

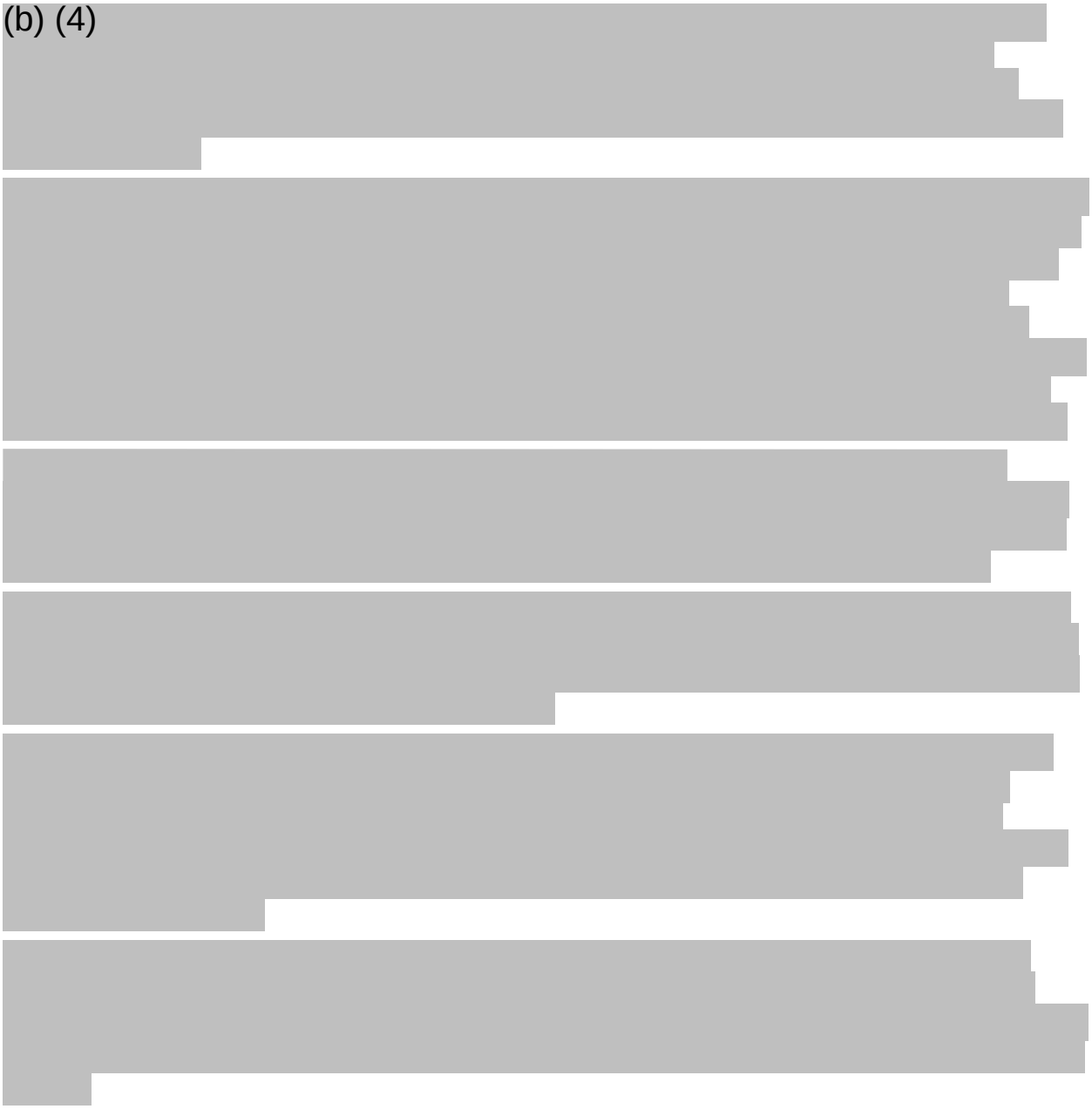
Reviewer's Assessment: *The validation of the filter sterilization, including heat distribution, heat penetration and biological indicator challenge studies appear appropriate.* (b) (4) *qualification is further reviewed during the PLI of February 21-24, 2023 and no issues were noted.*

Sterile Filtration Validation

A filter validation study was conducted to provide bacterial retention data for the sterilization filter used to (b) (4) drug product. (b) (4)

1 page determined to be not releasable: (b)(4)

(b) (4)



Environment Monitoring

The cleanroom manufacturing spaces for SRP-9001 include (b) (4) and Aseptic Fill areas. Each area is designed and qualified to meet requirements for Grades (b) (4) cleanroom classifications per (b) (4) standards.

The Environmental Monitoring Performance Qualification (EMPQ) was performed to evaluate the flow of personnel, materials, air as well as the gowning and sanitization practices throughout the manufacturing space. The qualification consisted of (b) (4) of static monitoring and (b) (4) of dynamic monitoring. Non-viable particulate sample sites were selected as outlined in (b) (4). All other environmental

monitoring sample sites were established based upon risk-based analysis of the EMPQ data.

Action limits for non-viable particulates and viable particulates are described in Table 16 and Table 17:

(b) (4)

(b) (4)

(b) (4)

(b) (4)

An EMPQ was performed during the HVAC requalification of (b) (4) , and no excursion was noted.

The routine environmental monitoring (EM) program consists of viable and non-viable particulate air sampling and viable surface sampling. Routine and in-process monitoring is performed at pre-defined frequencies to ensure a continued state of environmental control in the cleanrooms and BSCs. EM locations are selected according to the site's risk assessment. Surface sites were selected throughout the facility based upon worst-case locations, proximity to process, probability of operator contact, equipment locations or materials of construction. All data and facility environmental isolates are trended, analyzed, and summarized on a (b) (4) basis. EM monitoring frequency is summarized in Table 18.

(b) (4)

(b) (4)

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(b) (4)

(b) (4)

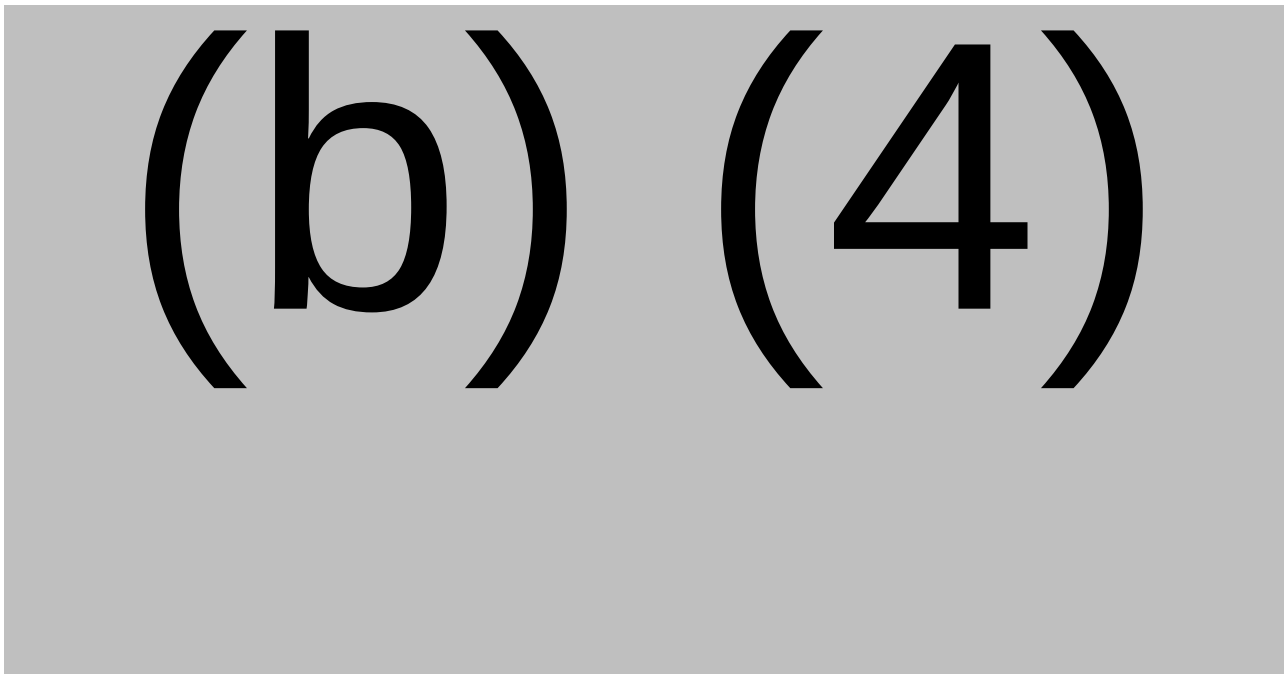
No EM excursion was reported.

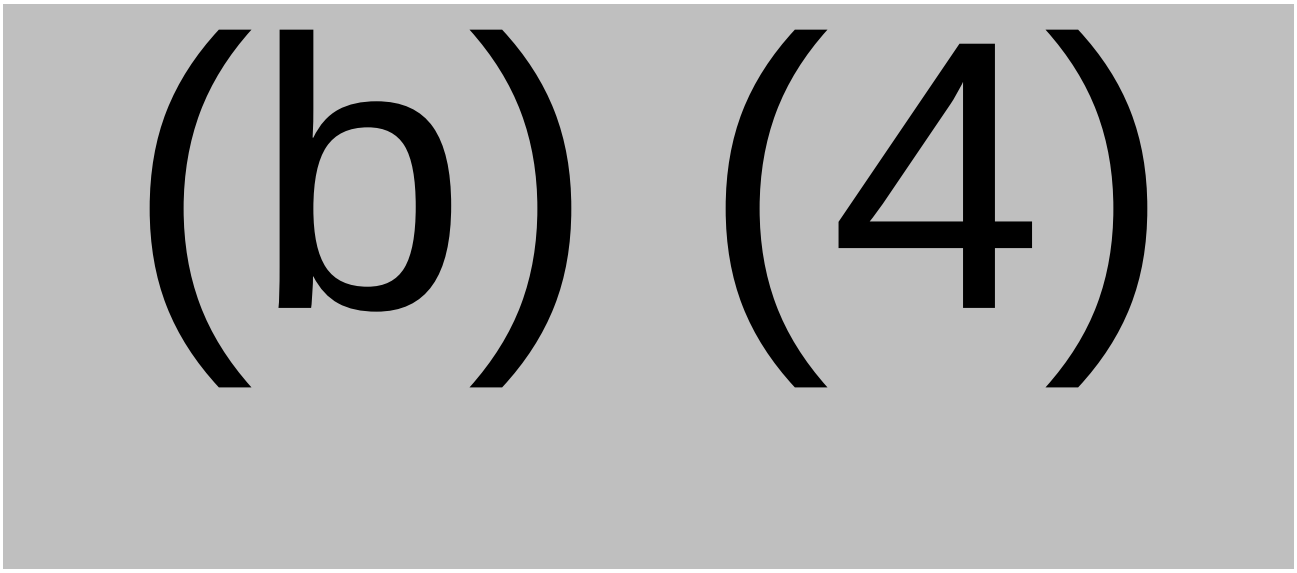
Reviewer's Comment: *The EMPQ and routine EM program appear acceptable. EM, including deviations, was reviewed in further detail during the PLI of February 21-24, 2023. Refer to Establishment Inspection Report for details.*

Aseptic Process Validation

At Catalent Biopark facility, initial Aseptic Process Qualification requires (b) (4)

(b) (4)





No growth was observed in any vials, and all growth promotion studies pass.

Reviewer's Assessment: *Catalent Biopark's media fill qualification and requalification program appear adequate, and the interventions are appropriately designed. SRP-9001 aseptic filling parameters and container specifications appear to be* (b) (4) *by the recent media fills,* (b) (4)

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(b) (4)

this appears acceptable.

Shipping Validation

(b) (4)

(b) (4)

(b) (4)

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(b) (4)

Reviewer's Assessment: *It appears acceptable for the shipping validation to focus on thermal control and package integrity, as product-specific attributes are conserved when stored at ≤ -60 °C as verified in the stability study. Temperature control, packaging integrity, and CCIT all met pre-defined acceptance criteria. Shipment from the manufacturing site Catalent BioPark to the finished goods packaging site* (b) (4) *as well as the finished goods packing site* (b) (4) *to the Specialty Pharmacy or End User appear to be appropriately qualified.*

3.2.R Regional Information (USA)

Comparability Protocol

(b) (4)

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(b) (4)

Exhibit G

CBER CMC BLA Review Memorandum

BLA STN 125781

delandistrogene moxeparvovec-rokl
ELEVIDYS

Reviewers

Lilia Lei Bi, Ph.D., Biologist, OTP/OGT/DGT1/GTB1
Emmanuel Adu-Gyamfi, Ph.D., Biologist, OTP/OGT/DGT1/GTB1
Sukyoung Sohn, Ph.D., Biologist, OTP/OGT/DGT1/GTB1
Brian Stultz, M.S., Biological Reviewer, OTP/OGT/DGT1/GTB3
Andrey Sarafanov, Ph.D., Chemist, OTP/OPPT/DH/HB2

1. BLA#: STN 125781

2. APPLICANT NAME AND LICENSE NUMBER
Sarepta Therapeutics, License No. 2308

3. PRODUCT NAME/PRODUCT TYPE

Non-Proprietary/Proper/USAN:	delandistrogene moxeparvovec-rokl
Proprietary Name:	ELEVIDYS
Company codename:	SRP-9001
UNII Code:	2P6QV2ZE52
NDC Code (vial):	60923-501-10

4. GENERAL DESCRIPTION OF THE FINAL PRODUCT

Pharmacological category:	Adeno-associated virus vector-based gene therapy
Dosage form:	Suspension for injection
Strength/Potency:	1.33 x 10^{13} vector genome copies (vg)/mL
Route of administration:	Intravenous infusion
Indication:	For the treatment of ambulatory patients aged 4 through 5 years with Duchenne muscular dystrophy (DMD) with a confirmed mutation in the DMD gene.

5. MAJOR MILESTONES

BLA Milestone	Dates
IND received from Dr. Jerry Mendell (Nationwide Children's Hospital)	5-Oct-17
IND transferred to Sarepta Therapeutics, Inc.	21-Sep-18
(b) (4)	(b) (4)
Fast Track designation granted	4-Jun-20
Type B End of Phase 2	27-Jul-21
Original BLA submission-Accelerated approval	28-Sep-22
First Committee Meeting	24-Oct-22
Filing Meeting	14-Nov-22
60-day filing date	25-Nov-22
Internal Mid-cycle Meeting	9-Jan-23
Mid-cycle Applicant t-con	24-Jan-23
Internal Late-cycle Meeting	21-Feb-23
Late-cycle Meeting	13-Mar-23
Original PDUFA Action Date	26-May-23
Extended PDUFA Action Date	22-June-23

6. CMC/QUALITY REVIEW TEAM

Reviewer/Affiliation	Section/Subject Matter
Lilia Lei Bi, PhD, OTP/OGT/DGT1/GTB1	DS manufacturing process and process validation/ DP manufacturing process and process validation/ Viral clearance study

Emmanuel Adu-Gyamfi, PhD, OTP/OGT/DGT1/GTB1	Elucidation of Structure and Other Characteristics/Impurities/Specification(s)/Justification of Specification(s)/Analytical methods and validation /Batch Analysis/Container closure/reference standard/ Physicochemical and Biological Properties/pharmaceutical development/Environmental assessment/Batch records/ Analytical Procedures for Assessment of Clinical and Animal Study Endpoints
Sukyoung Sohn, PhD, OTP/OGT/DGT1/GTB1	Control of materials/ Shipping validation/ DP manufacturer/ DP batch formular/ Control of excipients/ Stability
Brian Stultz, MS, OTP/OGT/DGT1/GTB3	Analytical Procedures for Assessment of Clinical Endpoint
Andrey Sarafanov PhD, OTP/OPPT/DH/HB2	Process / Storage Leachables assessment in DP

7. INTER-CENTER CONSULTS REQUESTED: N/A

8. SUBMISSION(S) REVIEWED

Date Received	Submission	Comments/ Status
9/28/2022	125781/0	Original submission
10/24/2022	125781/2	Response to DMPQ IR sent on 10/19/2022
12/6/2022	125781/4	Response to DMPQ IR sent on 11/29/2022
12/8/2022	125781/5	Response to DMPQ IR sent on 11/28/2022
12/22/2022	125781/6	Response to DMPQ IR sent on 12/14/2022
12/29/2022	125781/8	Response to CMC IR sent on 12/21/2022
1/11/2023	125781/12	Response to DBSQC IR sent on 1/6/2023
1/12/2023	125781/13	Response to DMPQ IR sent on 12/27/2022
1/19/2023	125781/14	Response to DMPQ IR sent on 1/17/2023
1/20/2023	125781/15	Response to DBSQC IR sent on 1/10/2023
1/24/2023	125781/16	Response to DMPQ IR sent on 1/10/2023
2/3/2023	125781/19	Response to CMC IR sent on 1/20/2023
2/17/2023	125781/20	Response to CMC IR sent on 2/16/2023
2/21/2023	125781/22	Response to CMC IR sent on 2/13/2023
3/1/2023	125781/24	Response to CMC IR sent on 2/13/2023
3/3/2023	125781/27	Response to CMC IR sent on 2/24/2023
3/17/2023	125781/29	Commitments in response to CMC IRs sent on 2/13/2023, 2/16/2023
4/14/2023	125781/38	Commitment in response to DMPQ IR sent on 12/14/2022

4/17/2023	125781/40	Response to CMC IRs sent on 4/12/2023, 4/13/2023
4/21/2023	125781/43	Commitment for PMC in response to CMC IR sent on 1/20/2023
4/21/2023	125781/44	Response to CMC IR sent on 4/13/2023
4/26/2023	125781/46	Response to DBSQC IR sent on 4/19/2023
4/27/2023	125781/47	Response to CMC IR sent on 4/20/2023
5/3/2023	125781/50	Response to CMC IR sent on 4/27/2023
5/10/2023	125781/52	Response to DBSQC IR sent on 5/8/2023, Updated Lot Release Protocol (LRP)
5/11/2023	125781/53	Response to CMC IR sent on 4/27/2023, DP Method Validation
5/16/2023	125781/54	Response to CMC IRs sent on 4/27/2023, 5/11/2023
5/19/2023	125781/59	Response to Form FDA 483 observations from the pre-license inspection of Catalent BWI
5/30/2023	125781/60	Response to CMC postmarketing commitments (PMCs) sent on 5/24/2023
6/7/2023	125781/66	Response to CMC IR sent on 6/2/2023
6/8/2023	125781/68	Response to CMC postmarketing commitments (PMCs) sent on 6/5/2023
6/14/2023	125781/70	Response to CMC IR sent on 6/12/2023
6/20/2023	125781/73	Response to CMC IR sent on 6/16/2023
6/15/2023	125781/75	Response to CMC IR sent on 6/14/2023
6/21/2023	125781/77	Response to Vial-Carton Label request sent on 6/20/2023

9. Referenced REGULATORY SUBMISSIONS (e.g., IND BLA, 510K, Master File, etc.)

Submission Type & #	Holder	Referenced Item	Letter of Cross-Reference	Comments/Status
DMF (b) (4)	(b) (4)	(b) (4) Vial	Yes	Information supports section (3.2.P.7.2) of BLA
DMF (b) (4)	(b) (4)	Vial Sterilization of (b) (4) vials	Yes	Information supports section (3.2.P.5.1) of BLA
MF (b) (4)	(b) (4)	Sterilization validation results for Stopper	Yes	Information supports section (3.2.P.5.2) of BLA
STN (b) (4)	(b) (4)	Stopper, elastomeric formulations, coatings, films	Yes	Section 3.2.P.7

10. REVIEWER SUMMARY AND RECOMMENDATION

A. EXECUTIVE SUMMARY

Based on the review of the collective CMC information submitted in the BLA by the Applicant and subsequent information requests reviewed throughout the review period, the CMC review team concludes that the manufacturing and controls for delandistrogene moxeparvovec-rokl (also referred to as SRP-9001; ELEVIDYS) are capable of yielding the drug product with consistent quality attributes and therefore, deemed acceptable for commercial manufacturing under the accelerated approval for this BLA.

Description of the product: SRP-9001 (rAAVrh74.MHCK7.micro-dystrophin) consists of a 4.7 Kb codon-optimized DNA vector genome encapsidated in a simian AAV serotype rh74 capsid. Each virion potentially contains a single copy of the vector genome. The vector genome expresses micro-dystrophin (µ-Dys), a novel, engineered protein consisting of select domains from the full-length dystrophin protein, which are essential for muscle contractions and turnover. The vector genome expression cassette contains essential elements to control gene expression, including AAV2 inverted terminal repeats (ITRs), a chimeric (SV40) intron, and a synthetic polyadenylation (Poly A) signal (See Figure 1). Expression of the micro-dystrophin protein is under the control of the α-myosin heavy-chain creatine kinase 7 (MHCK7) promoter to restrict expression to skeletal and cardiac muscle.

Manufacturing and process validation:

(b) (4)

The delandistrogene moxeparvovec drug product (DP) is manufactured at the Catalent Pharma Solutions facility (BioPark), Baltimore, Maryland. Each DP vial contains an extractable volume of not less than 10 mL, with a nominal concentration of 1.33×10^{13} vector genome (vg)/mL formulated in 7 mM Tromethamine / 13 mM Tromethamine HCl, 200 mM Sodium chloride, 1 mM Magnesium chloride, 0.001% Poloxamer 188, at (b) (4) . The DP manufacturing process includes formulation buffer preparation, (b) (4) , sterile filtration, aseptic filling, stoppering, and capping. After visual inspection, the vials are packed, stored at (b) (4), and shipped to the labeling and secondary

packaging site. Validation of the DP manufacturing process included three PPQ runs. The DP manufacturing process is validated for commercial manufacturing.

The manufacturers accept raw materials based on specified quality attributes, including (b) (4) Raw materials derived from animals are appropriately controlled to ensure the absence of microbial contaminants.

Control and testing: The manufacturing steps, (b) (4), and final DP are controlled and characterized by a panel of analytical methods that are used for characterization and release. These include quantitative assays that assess critical measures of product quality, safety, purity, strength (vg/mL), and potency attributes. The potency test measures the ability of the SRP-9001 to successfully transduce a dystrophin (b) (4) and express the miniaturized micro-dystrophin, which is measured via quantitative (b) (4). There is a (b) (4) potency tests that ensure the (b) (4) of the microdystrophin to the (b) (4) Collectively, the assays used as part of the overall controls for the manufacturing process were found to be fit-for-purpose. Release and characterization test methods are discussed in detail in this BLA memo.

Stability: The DS is stable for (b) (4) when stored at the long-term storage condition of (b) (4). The DP is stable for 12 months at the storage condition of ≤-60°C. During administration of the DP in the clinic, the DP is thawed and aspirated into an infusion syringe to be infused with a syringe pump. Based on the stability data submitted in the BLA, the thawed DP is stable for up to 24 hours at room temperature (15°C to 25°C) and stable for up to 14 days at 2°C to 8°C.

Comparability: Two manufacturing processes were utilized to generate purified DP to support the clinical program. For early clinical trials (Study SRP-9001-101 and Study 102), the DP was made using manufacturing **Process A** at Nationwide Children's Hospital (Ohio State University). Process A used a (b) (4)-based purification process to achieve a near complete removal of empty AAV capsids from the final formulated product. For late-stage clinical trials (Study SRP-9001-103 and ongoing Phase 3 trial [Study 301]), the DP was purified using the to-be-commercialized manufacturing process, referred to as **Process B** at Catalent Pharma Solutions (Baltimore, MD). Process B utilizes a scaled-up purification method that incorporates chromatography-based methods purification of the DP, including separation of the empty capsid residuals from the full capsids. The Process B purification method results in less efficient separation of empty AAV capsids from full AAV capsids (b) (4) full capsids), which contain the SRP-9001 micro-dystrophin DNA.

Based on both the Applicant's and FDA's assessment, it was concluded that the Process A and Process B materials are not analytically comparable relative to the levels of empty capsid residuals. The percent (%) full capsids of Process A and Process B material were found to be significantly different with a statistical probability t-test with p value = 0.0002.

B. RECOMMENDATION

I. APPROVAL

This biological license application (BLA) provides an adequate description of the manufacturing process and characterization of the new drug product delandistrogene moxeparvovec-rokl to support accelerated approval. The CMC review team has concluded that the manufacturing process, along with associated test methods and control measures, can yield a product with consistent quality attributes. This information, along with post-marketing commitments (PMC) from Sarepta, fulfills the CMC requirements for biological product licensure per the provisions of

section 351(a) of the Public Health Service (PHS) Act controlling the manufacture and sale of biological products and thus, we recommend approval under the accelerated approval pathway requested by the Applicant. When the confirmatory study is completed, the applicant will submit additional CMC data that may require revision of some aspects of this approval memo (e.g., specifications, etc based on additional manufacturing data).

Post-Marketing Commitments (PMCs):

1. Sarepta Therapeutics, Inc. commits to performing (b) (4) as a ‘‘Postmarketing Commitment- Final Study Report” by July 31, 2024

2. Sarepta Therapeutics, Inc. commits to submitting a final report for the supplemental (b) (4) manufacturing runs for (b) (4) at the Catalent facility as a “Postmarketing Commitment - Final Study Report” by June 30, 2024.

3. Sarepta Therapeutics, Inc. commits to submitting a final report of the (b) (4) as a “Postmarketing Commitment - Final Study Report” by March 31, 2024.

4. Sarepta Therapeutics, Inc. commits to revising the system suitability criteria set in the SOP for (b) (4) to reflect the assay variability (percent coefficient of variation; %CV) observed in intermediate precision during assay validation and to submitting the revised SOP as a ‘‘Postmarketing Commitment - Final Study Report’’ by December 31, 2023

5. Sarepta Therapeutics Inc. commits to revising the system suitability in the SOP for the (b) (4) assay to include a parameter determining (b) (4) and to submitting the revised SOP as a “Postmarketing Study Commitment – Final Study Report” by June 30, 2024.

6. Sarepta Therapeutics Inc. commits to reassessing the commercial acceptance criterion for the release testing of potency of SRP-9001 drug product after data have been collected on (b) (4) commercial lots and submit a “Postmarketing Study Commitment – Final Study Report” by June 30, 2024.

7. Sarepta Therapeutics Inc. commits to implementing the following CMC change for the SRP-9001 (b) (4) . The CMC change will be submitted as a “Postmarketing Commitment - Final Study Report” by December 31, 2024.

8. Sarepta Therapeutics Inc. commits to performing (b) (4) . The final report will be submitted as a “Postmarketing Study Commitment – Final Study Report” by December 31, 2024.

II. COMPLETE RESPONSE (CR)

Clinical and clinical pharmacology Review teams are recommending complete response, due to their assessment that the data provided for the accelerated endpoint, micro-dystrophin expression, does not meet the requirement that this endpoint is reasonably likely to predict clinical benefit. This decision was overruled by Dr. Peter Marks, Director CBER and he approved this accelerated endpoint, and the BLA.

III. SIGNATURE BLOCK

Reviewer/Title/Affiliation	Concurrence	Signature and Date
Lilia Lei Bi, Ph.D. Biologist OTP/OGT/DGT1/GTB1	Concur	
Emmanuel Adu-Gyamfi, Ph.D. Biologist OTP/OGT/DGT1/GTB1	Concur	
Sukyoung Sohn, Ph.D. Biologist OTP/OGT/DGT1/GTB1	Concur	
Brian Stultz, M.S. Biological Reviewer OTP/OGT/DGT1/GTB3	Concur	
Andrey Sarafanov, Ph.D. Chemist OTP/OPPT/DH/HB2	Concur	
Andrew Harmon, Ph.D. Lead Biologist OTP/OGT/DGT1/GTB1	Concur	
Denise Gavin, Ph.D. Chief, Gene Therapy Branch 1 OTP/OGT/DGT1	Concur	
Kimberly L.W. Schultz, Ph.D. Chief, Gene Therapy Branch 4 OTP/OGT/DGT2	Concur	
Denise Gavin, Ph.D. Director, Office of Gene Therapy OTP/OGT	Concur	

Review of CTD

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3.2.S DRUG SUBSTANCE

3.2.S.1.1 - 1.3 Nomenclature, Structure and General Properties

Reviewed by Emmanuel Adu-Gyamfi (EAG)

Nomenclature

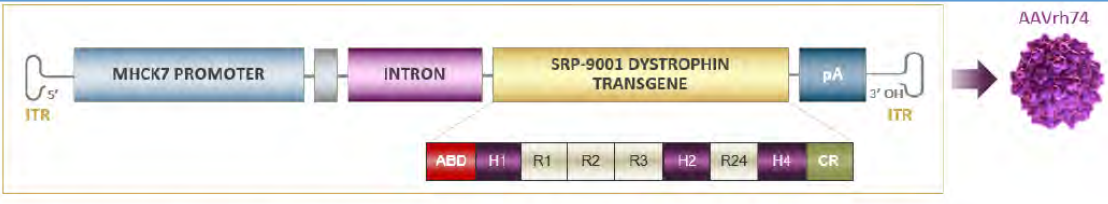
Table 1: Nomenclature of SRP-9001

International Nonproprietary Name (INN)	delandistrogene moxeparvovec-rokl
United States Adopted Name (USAN)	delandistrogene moxeparvovec-rokl
Proprietary name:	ELEVIDYS
Company code	SRP-9001
Chemical Abstracts Service (CAS) registry number	2305040-16-6
Chemical name (CAS Index Name)	DNA (Recombinant adeno-associated virus AAVrh74 vector SRP-9001 MHCK7 promoter plus micro-dystrophin-specifying)
Unique Ingredient Identifier (UNII)	2P6QV2ZE52
World Health Organization (WHO) Number	11631
Other Names	Micro-dystrophin, SRP-9001-micro-dystrophin

Structure

The drug substance (DS) vector genome is 4.7 kb in size. It is encapsidated in a rhesus AAV serotype rh74 capsid, with each virion potentially containing one copy of the viral genome. The vector genome contains a codon-optimized microdystrophin transgene (b) (4) derived from minimal elements of the full length wild-type human dystrophin gene. The vector genome also contains genetic elements required for gene expression, including AAV2 inverted terminal repeats (ITR), chimeric (SV40) intron, and synthetic polyadenylation (Poly A) signal, all under the control of the α-myosin heavy-chain creatine kinase 7 (MHCK7) promoter to restrict expression to skeletal and cardiac muscles. (b) (4) . Elements of the vector genome are schematically summarized under Figure 1and discussed under Table 2

Figure 1: SRP-9001 Vector Design



ABD = actin-binding domain; CR = cysteine-rich region; H = hinge; ITR = inverted terminal repeats; MHCK7 = α-myosin heavy-chain creatine kinase 7; pA = polyadenylation signal; R = spectrin-like repeat; rAAV = recombinant adeno-associated virus; rh74 = rhesus serotype 74.

General Properties

The SRP-9001 vector expresses a miniaturized version of the full-length dystrophin protein described above. This protein is truncated from the mild Becker Dystrophin (see Figure 2) used

to design the expression cassette. The dystrophin elements selected as well as the Applicant's rationale for the design of SRP-9001 construct is summarized under Table 2

Table 2: SRP-9001-micro-dystrophin vector elements

(b) (4)

(b) (4)

.

(b) (4)

(b) (4)

(b) (4)

3.2.P DRUG PRODUCT

3.2.P.1 Description and Composition of the Drug Product

Reviewed by LB

The SRP-9001 Drug Product (DP) is a sterile suspension for intravenous (IV) administration, containing 1.33×10^{13} vg/mL of delandistrogene moxeparvovec formulated in a buffered solution of Tromethamine/Tromethamine-HCl, Magnesium chloride, Sodium chloride, and Poloxamer 188. Each vial contains an extractable volume of not less than 10.0 mL. The total recommended dosage is based on patient weight and requires multiple vials per dose.

Table 77: Composition of the SRP-9001 Drug Product

Component	Quantity per 10 mL[a]	Concentration	Reference to Standard(s)	Function
Delandistrogene moxeparvovec	1.33×10^{14} vector genomes (vg)	1.33×10^{13} vector genomes (vg)/mL	In-house specification[b]	Active ingredient
Sodium chloride	(b) (4)	200 mM	(b) (4)	(b) (4)
Tromethamine HCl (b) (4)	(b) (4)	13 mM	In-house specification[c]	Buffer agent

Tromethamine (b) (4)	(b) (4)	7 mM	(b) (4)	Buffer agent
Magnesium chloride (Magnesium chloride (b) (4)	(b) (4)	1 mM	(b) (4) .	(b) (4)
Poloxamer 188	(b) (4)	0.001% (b) (4)	(b) (4) .	(b) (4)
(b) (4)	(b) (4)	q.s.	(b) (4)	(b) (4)

[a] Vial contains a target overfill of (b) (4) mL per vial to allow complete withdrawal of 10.0 mL dose.
[b] Specification is provided in Section 3.2.S.4.1.
[c] Manufactured for supplier under GMP using Tromethamine, (b) (4) is provided in Section 3.2.P.4.1 – Tromethamine HCl / (b) (4)
q.s. = quantity sufficient to achieve final volume

3.2.P.2 Pharmaceutical Development
3.2.P.2.1 Components of the Drug Product
3.2.P.2.1.1 Drug Substance
The drug substance (delandistrogene moxeparvovec) is (b) (4) in order to prepare the drug product. The drug substance is stored at (b) (4)

3.2.P.2.1.2 Excipients
The drug product is formulated in 7 mM Tromethamine (b) (4) / 13 mM Tromethamine HCl (b) (4) 200 mM Sodium chloride, 1 mM Magnesium chloride (Magnesium chloride (b) (4) 0.001% Poloxamer 188, at (b) (4)

3.2.P.2.2 Drug Product
3.2.P.2.2.1 Formulation Development
The components of the formulation are commonly used as ingredients of intravenous formulations and were selected to provide stability and compatibility of the drug product for the intended route of administration.

Development of SRP-9001 DP formulation was based on available knowledge of physicochemical properties of recombinant AAV serotypes. The same formulation has been used from initial clinical trials through to the commercial product.

3.2.P.2.2.2 Overages
There are no overages in the formulation of the DP.

3.2.P.2.2.3 Physicochemical and Biological Properties
Reviewed by EAG
The composition of the DS (b) (4) DP titer of 1.33 e13 vg/mL. According to the Applicant, the quality target product profile (QTPP) for the SRP-9001 program was compiled by a cross-functional team which included input from gene therapy research, process development, formulation development and analytical development, provides a comprehensive listing of the desired drug substance (DS)/ drug product (DP) quality attributes for the finished product. Subsequent assessment of critical quality attributes (CQAs) was performed. The QTPP for SRP-9001 is summarized below under Table 78.

Table 78: SRP-9001 Quality Target Product Profile

Category of Attribute	Quality Target Product Profile
Drug Product Attributes (General)	
Description	SRP-9001-micro-dystrophin is a recombinant gene therapy product designed to deliver the gene encoding the SRP-9001-micro-dystrophin protein. It is a non-replicating, recombinant, adeno-associated virus (AAV) serotype rh74 (AAVrh74) based vector containing the SRP-9001-micro-dystrophin expression cassette, under the control of the MHCK7 promoter.
Intended use in clinical setting	Adeno-associated virus gene therapy for the treatment of patients aged 4 through 5 with a confirmed diagnosis of Duchene Muscular Dystrophy (DMD).
Dosage form	SRP-9001 1.33 × 10^{13} vg/mL suspension for infusion is supplied as a single-use, clear to opalescent, colorless, preservative-free, sterile, aqueous solution for intravenous infusion, that may contain white to off-white particles. Multiple vials will be thawed and pooled at the clinical site and prepared for I.V. infusion to achieve the therapeutic dose per patient body weight in kg (1.33E14 vg/kg).
Route of administration	Intravenous. The product will be administered as a single IV infusion.
Delivery system	Microbore infusion set with syringe pump
Dose/Dose frequency	1.33E14 vg/kg, SRP-9001 administered as single (one time) peripheral venous infusion.
Container	SRP-9001 drug product is stored as a sterile frozen liquid formulation in a cyclic olefin polymer vial closed with a rubber stopper and sealed with an aluminum seal and plastic flip-off cap.
Shelf life	drug product shelf-life is 12 months.
Formulation biocompatibility	(b) (4) formulation buffer, containing generally regarded as safe (GRAS) excipients (20 mM Tris, 200 mM NaCl, 1 mM MgCl2, (b) (4) 0.001% (b) (4) Poloxamer 188.
Primary sequence and therapeutic moiety integrity	SRP-9001-micro-dystrophin is a recombinant gene therapy product designed to deliver the gene encoding the 9001-micro-dystrophin protein. It is a non-replicating, recombinant, adeno-associated virus (AAV) serotype rh74 (AAVrh74) based vector containing the 9001-micro-dystrophin expression cassette, under the control of the MHCK7 promoter.

Reviewer Comment:

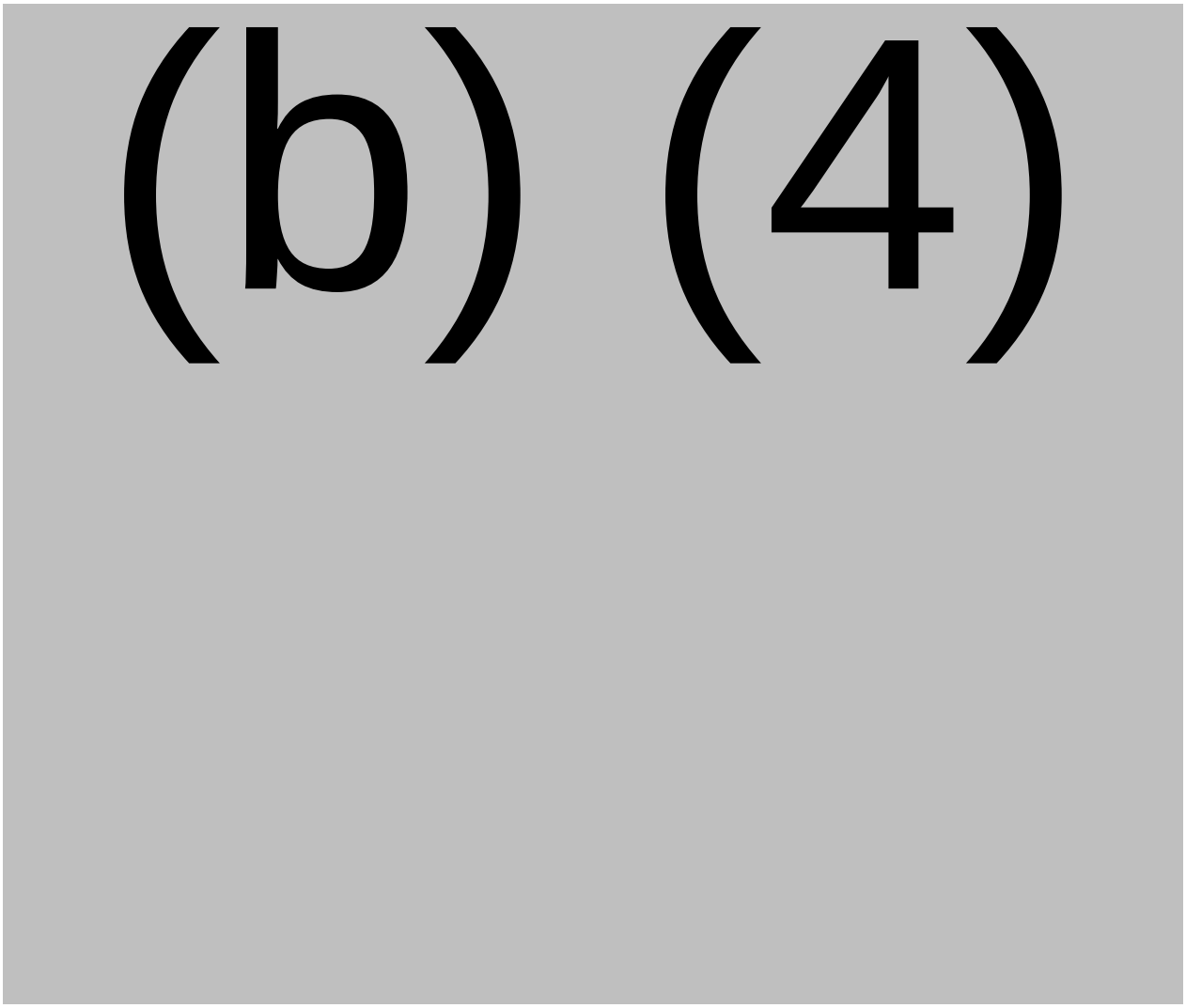
- *The QTPP does not fully specify Drug product quality criteria (e.g., sterility, purity, stability, and drug release) appropriate for an intended marketed product. However, these are clearly indicated in release specification.*
- *Although i(b) (4) guideline on QTPP summary is not explicit about content, the QTPP should incorporate a summary on handling and storage as this is critical to product and process quality. Table 78 reflects the revised QTPP table submitted by the Applicant under Amendment #70 submitted 2023.06.14. This is acceptable.*

Assessment of SRP-9001 Product Criticality

Critical quality attributes (CQAs) for the drug substance and drug product have been designated based on product and process development experience, nonclinical, as well as published literature and publicly available information on other AAV product (CQAs) were evaluated in process development and characterization studies. Manufacturing process steps were evaluated to understand their impact on CQAs, and relevant methods were chosen for specific studies evaluating. safety, purity, potency and identity. *These have been discussed and summarized under 3.2.P.3.5 Process Validation* and/or Evaluation. CQAs are grouped mainly under safety, purity, potency and identity See table below. Purity was further divided into

process and product related impurities. *Note: Practically, all attributes reported on release CoA are classified as CQA and are monitored at release or as in-process steps.*

Table 79: Categorization of critical quality attributes



Reviewer Comment: *The rationale for criticality assignments did not include any dedicated criticality scoring scheme that considers all the information gathered from manufacturing, known safety concerns in the literature as well as regulatory and compendial requirements. However, the assignments of product criticality is consistent with current understanding of AAV products in the field. Also, practically every measured attribute reported on release is considered critical and is thus monitored accordingly. Hence, this is acceptable.*

3.2.P.2.3 Manufacturing Process Development

Reviewed by EAG and AS

Two manufacturing processes have been utilized during the clinical trial stages for SRP-9001 program: early clinical manufacturing Process A and late-stage Process B. *The comparability study to support the drug product Process A to Process B manufacturing change already discussed under 3.2.S.2.6 Manufacturing Process Development.* Process B clinical DP is manufactured at Catalent BioPark and has been validated as the intended commercial process, while Process A was not validated.

3.2.P.2.4 Container Closure System

Reviewed by EAG and Andrey Sarafanov (AS)

Primary Container: SRP-9001 is supplied in a 10-mL (b) (4) cyclic olefin polymer (b) (4) vial with a rubber stopper and capped with an aluminum seal. According to the Applicant, all components are received in pre-sterilized, ready-to-use configurations. The components and schematic of the container closure system (CCS) are summarized under Figure 21 and Table 80 below.

Figure 21: Schematics of primary container closure of SRP-9001

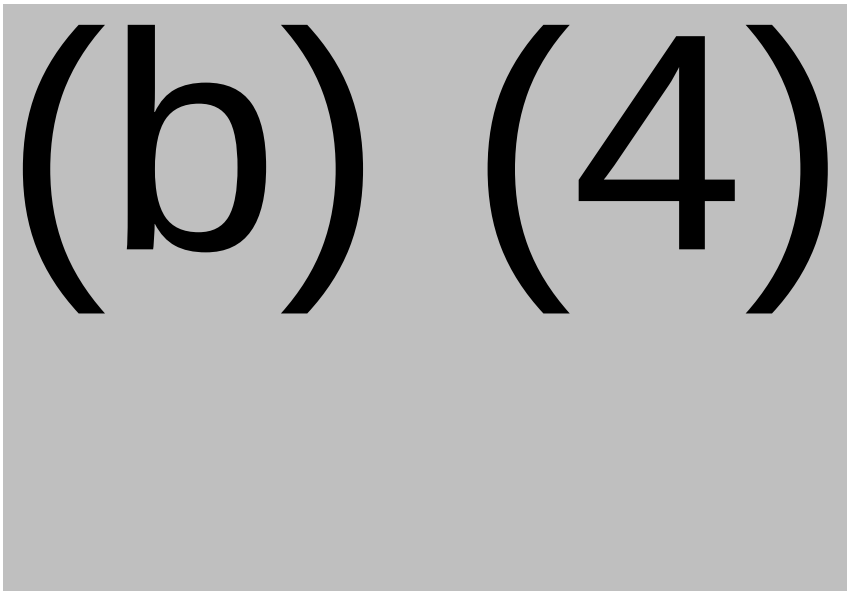


Table 80: Summary of DP primary container closure description

Component	Description	Manufacturer	MF/DMF[a]	Reference to Standards
Vial	10-mL, cyclic olefin polymer (b) (4)	(b) (4) .	(b) (4) (letter of authorization to DMF (b) (4)	(b) (4)
Stopper	20-mm, (b) (4) grey chlorobutyl rubber stopper with (b) (4) barrier on product contact side, (b) (4) on non-product side	(b) (4) .	STN (b) (4) (letter of authorization to DMF STN (b) (4)	(b) (4)
Seal	20-mm, aluminum shell with polypropylene flip- off cap overseal	(b) (4)	Not applicable (no product contact)	Not applicable (no product contact)

[a] DMFs containing the sterilization information for components listed in the table are located in Section 3.2.P.3.5.
[b] (b) (4)

(b) (4)

Container Closure System Extractable and Leachables Substances Determination

(b) (4)

Secondary Packaging: The secondary packaging consists of an opaque, tamper-evident, rigid paperboard carton. The carton provides physical protection for the vials during storage and shipping. *Reviewer Note: During PLI for the DP (at Catalent-BioPark, Baltimore MD), the secondary packaging container was inspected to be acceptable. Details can be found in the EIR report for the drug product manufacturing site.*

3.2.P.2.5 Microbiological Attributes

Reviewed by EAG

SRP-9001 DP is manufactured by aseptic processing, for intravenous infusion to avoid microbial contamination. The DP is supplied as single-use vials free of preservative. As part of processing, DP solution is filtered through (b) (4) filters. DP is aseptically filled using a process that has been validated. Components that have direct contact with the DP are either received sterile or sterilized during the process. DP is subject to sterility and endotoxin testing as part of the release process. For assurance of container closure system (CCS) integrity, the DP vial is tested for CCS integrity during stability testing, in lieu of sterility testing via (b) (4) method and has been developed according to the principles in (b) (4). *This is acceptable. Also, for detailed microbial containment strategy see DBSQC memo.*

3.2.P.2.6 Compatibility

Reviewee by EAG

SRP-9001 drug product (DP) is supplied as single-use, preservative-free, sterile, aqueous solution for infusion, to be administered with a 0.2 µm in-line dosing filter. Studies to assess for potential change in (b) (4) were performed during clinical development. (b) (4) independent compatibility studies were performed using representative DP (lot (b) (4) in which the DP was (b) (4)

Dosing Filter Compatibility Study (Study 3)

According to the Applicant, low levels of visible particles were observed in SRP-9001 drug product vials during the 100% visual inspection process in some batches and were rejected. Based on investigations conducted, the Applicant concluded that the formulated DP has the propensity to also form inherent (b) (4) -related particles. Therefore, as a risk mitigation strategy, a dosing filter is needed in the infusion line to reduce the level of inherent subvisible and visible particles in the dosing solution. A study to assess the in-use compatibility and effectiveness of a 0.2 µm in-line filter as part of DP administration to remove potential intrinsic particulates in the DP, was conducted using the delivery device components listed in the table below. (*Note: delivery device components were requested during BLA review under Amendment # 27, 2023.03.03). The Applicant also clarified that the in-line filter was introduced in Study SRP-9001-103 and the ongoing pivotal trial (SRP-9001-301). Clinical studies SRP-9001-101 and 102 did not use the in-line filter for administration.*

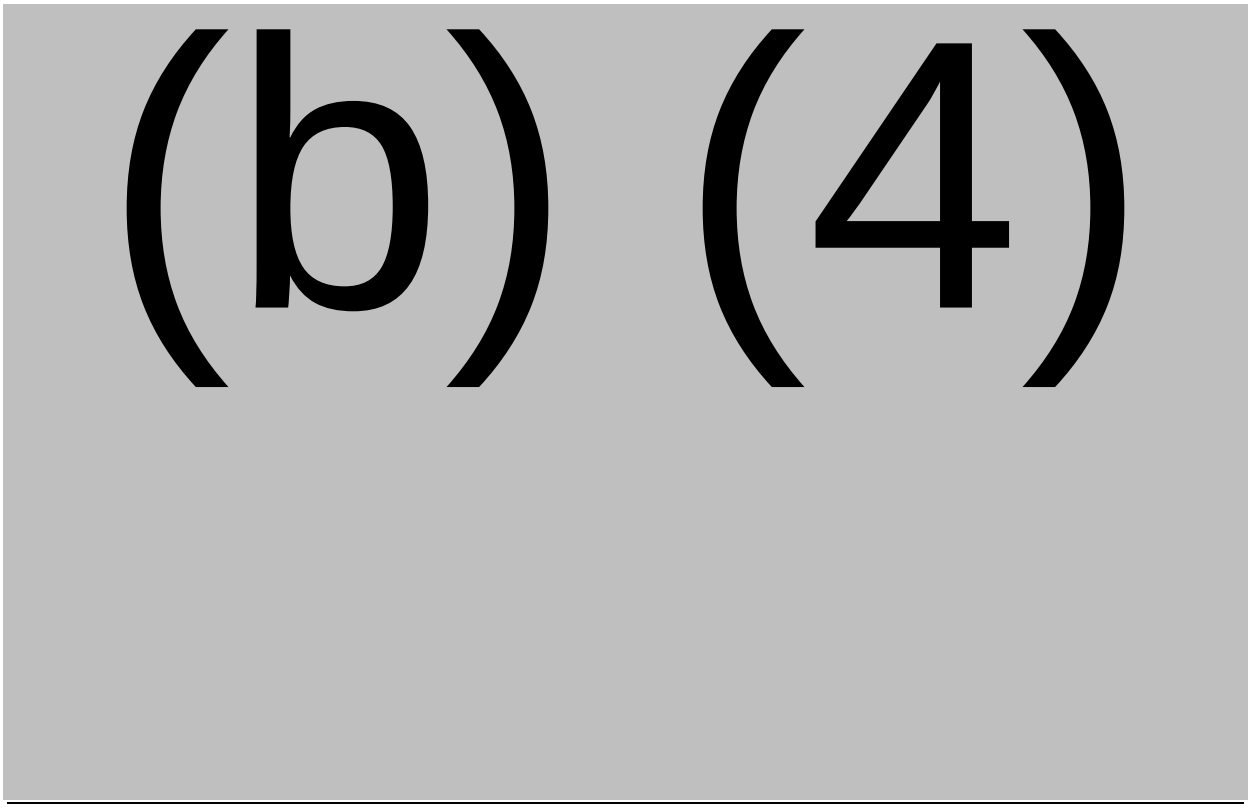
Table 81: Description of Delivery Device Set Used in Compatibility Studies

Component	Description	Manufacturer	Part Number	Sarepta In-Use Compatibility Study Number	Material of Construction
Study 3					

Syringe	60 mL plastic syringe (b) (4)	(b) (4)	(b) (4)	RPT-02108	Siliconized polypropylene
IV infusion extension set	Non-DEHP IV infusion extension	(b) (4)	(b) (4)		PVC (non-DEHP)
IV catheter	(b) (4) Catheter	(b) (4)	(b) (4)		Polyurethane
Needle	21-G Precision Glide needle	(b) (4)	(b) (4)		Stainless steel
In-line dosing filter	Non-DEHP in-line filter extension set (0.2 µm PES filter)	(b) (4)	(b) (4)		PVDF (hydrophobic air vent filter membrane), Copolyester (housing material), PVC (non-DEHP, tubing), PES (filter)
DEHP = Di(2-ethylhexyl)phthalate PES = polyethersulfone PVC = polyvinyl chloride PVDF = (b) (4)					

(b) (4)

(b) (4)



(b) (4)

Reviewer Comment:

- (b) (4)

Overall Reviewer's Assessment of Section 3.2.P.2:

- ❑ Information provided to describe pharmaceutical development together with the additional IR responses are acceptable.
- ❑ State if deficiencies were identified and how they were resolved.
  - Limited information regarding description of the delivery device components used in the assessment of device compatibility study was present in the BLA. This was resolved with the Applicant through an IR request (Amendment 27).
  - Absence of assessment of cumulative process leachables in DP. Upon FDA request, the Applicant committed performing such assessment (PMC #1). The study details, recommended by FDA (see review memo of Dr. Andrey Sarafanov), were communicated to the Applicant on January 20, 2023 (Question 24).

3.2.P.3 Manufacture

3.2.P.3.1 Manufacturer(s)

DP Manufacturers are summarized the table below.

Table 83: Description of DP Manufacturers

Site Name and Address	FEI	DUNS	Responsibility	Testing Performed (if applicable)
Catalent Pharma Solutions[a] Catalent Maryland (BioPark) 801 West Baltimore Street, Suite 302, Baltimore, MD 21201, USA	3015558590	618890289	Manufacture	(b) (4), Bioburden, Filter integrity, Fill weight check
(b) (4)	(b) (4)	(b) (4)	Labeling Secondary packaging	n/a

(b) (4)			DP storage	
(b) (4)	(b) (4)	(b) (4)	Release testing Stability testing	Release: All methods except Vector genome concentration and Potency Stability: All methods except Vector genome concentration, Potency, and Container closure integrity test
(b) (4)	(b) (4)	(b) (4)	Release testing	All methods except Vector genome concentration, Potency, Percent full capsid, and Identity (vector capsid)
Sarepta Therapeutics 100 Federal Street, Andover, MA 01810, USA	3012807588	072827382	Release testing Stability testing	Potency and Vector genome concentration
(b) (4)	(b) (4)	(b) (4)	Stability testing	Container closure integrity test
[a] Previously Paragon Bioservices, Inc.				

Reviewer's comment: *The updated list of DP manufacturers was submitted under Amendment #19 dated 02/17/2023.*

3.2.P.3.2 Batch Formula

Each DP batch is prepared according to the formula summarized in Table 84. A batch consists of (b) (4) (approximately (b) (4) vials) of pre-sterilized DP.

Table 84: DP Batch Formula

Component	Quantity	
	Min Batch Size (b) (4)	Max Batch Size (b) (4)
Delandistrogene moxeparvovec	(b) (4)	(b) (4)
Sodium chloride		
Tromethamine HCl (b) (4)		
Tromethamine (b) (4)		
Magnesium chloride (Magnesium chloride (b) (4)		
Poloxamer 188		
(b) (4)		

[a] Target DP titer of 1.33×10^{13} vg/mL multiplied by the batch (b) (4), is presented. The total vg per batch for each DP formulation is calculated from the (b) (4), which is determined by (b) (4) during DS release testing.
q.s. = quantity sufficient to achieve final (b) (4)

Overall Reviewer's Assessment of Sections 3.2.P.3.1 and 3.2.P.3.2:

- ❑ Descriptions of DP manufacturers and the DP batch formula are acceptable.

1 page determined to be not releasable: (b)(4)

(b) (4)

- **Visual Inspection**

All filled vials are manually inspected for container closure and solution defects. The inspection process is qualified to remove container integrity defects which may compromise the sterility of the product, and to remove other container and product characteristic defects such as variations in fill level, discoloration or clarity. Vials found to have defects, including visible particles are removed. Vials passing the 100% manual visual inspection process are then sampled for an Acceptable Quality Limit (AQL) visual inspection.

- **Freezing and Storage**

The drug product vials are frozen and stored at ≤ -60°C.

- **Labeling and Packaging**

Drug product is transferred to the secondary packaging site. Labeling and packaging operations are performed at (b) (4) under GMP on qualified equipment according to standard operating procedures.

Long-Term Storage: The final drug product vials are stored at ≤ -60°C.

Reprocessing: No reprocessing is allowed during the manufacturing of the drug product.

Reviewer Comment: *In response to IRs, the Applicant provided information on DP labeling and packaging in amendment #19 of 03Feb2023 and in amendment #27 of 03Mar2023. The provided information is acceptable.*

DP Labeling and Packaging

The unlabeled vials are shipped from Catalent BioPark to (b) (4) for vial labeling and secondary packaging. In summary, after the drug product vials are manufactured and inspected, they are packaged directly into (b) (4) . These (b) (4)

The Primary (vial) and Secondary (carton) Labeling Procedures:

Drug product vials are labeled in a room maintained at (b) (4) . The primary vial labels are manually applied to the vials and visually inspected. Labeled vials are transferred into (b) (4) secondary carton. The carton is sealed with tamper evident seals to maintain product identity. Finished goods are (b) (4) . The established labeling procedures and controls are supported by the (b) (4) study summarized under stability section 3.2.P.8 Stability).

Identity testing by (b) (4) is performed at (b) (4) to ensure identity of the product in accordance with 21CFR 610.14. Confirmation of the passing ID test by QA is required. The labeled DP is packed and shipped for distribution (of the commercial finished goods) to Sarepta customers from the same packaging site (b) (4) and will only be sent to the clinical site when a DP administration is scheduled. Drug product will not be stored at the dosing site long term. These commercial shipments are completed using qualified, temperature-controlled shipping processes and shippers that utilize dry ice to maintain temperature. Temperature is continuously monitored for these shipments using specialty logistics courier. Instructions for the storage of the product at the dosing sites are provided in the USPI to customers. *Reviewer Comment: Description of labeling and shipping procedures are acceptable.*

Overall Reviewer's Assessment of Section 3.2.P.3.3:
The description of DP manufacturing process is acceptable.
In response to IRs, the Applicant provided the information on DP labeling and packaging in amendment #19 of 03Feb2023 and in amendment #27 of 03Mar2023. The provided information is acceptable.

3.2.P.3.4 In-process Controls

Reviewed by LB

Throughout the SRP-9001 manufacturing process, multiple process parameters are controlled and monitored. The controls of the critical steps of the commercial manufacturing process for SRP-9001 Drug Product are summarized in following tables:

Table 86: Critical Process Parameters in SRP-9001 DP Manufacture

(b) (4)

Table 87: In-Process Controls for SRP-9001 DP

(b) (4)

(b) (4)

Table 88: DP In-Process Hold Times

(b) (4)

(b) (4)

3.2.P.3.5 Process Validation and/or Evaluation

Reviewed by LB and AS

Process Validation Overview

The Applicant states that the Process Validation of the SRP-9001 drug product (DP) manufacturing process has been carried out with adherence to current regulatory guidelines. Process validation is executed through a methodical sequence of cross-functional activities that incorporate the evolving knowledge of the drug product and its characteristics with an understanding of the production process gained through experimentation, experience and GMP manufacturing activities. The process validation lifecycle links product and process development, validation of the commercial manufacturing process and on-going verification to ensure the process remains in a state of control throughout routine commercial production. The process validation program follows written guidelines aligned with ICH guidance. The various aspects of process qualification include:

- (b) (4) studies

8 pages determined to be not releasable: (b)(4)

Overall Reviewer's Assessment of Section 3.2.P.3.5:
The DP manufacturing process validation results generated from the PPQ runs are acceptable. The results of (b) (4) will be submitted by March 31, 2024 – PMC #3.

3.2.P.4 Control of Excipients

Reviewed by SS

3.2.P.4.1 Specifications

Compendial excipient

The compendial excipients are summarized in Table 98 and comply with the quality standards as referenced in Table 99.

Table 98: Compendial Excipients

Excipient	Complies with
Sodium chloride	(b) (4)
Tromethamine (b) (4)	
Magnesium chloride (Magnesium chloride (b) (4)	
Poloxamer 188	
(b) (4)	

Non-compendial excipient

Tromethamine HCl ((b) (4) is a non-compendial excipient which is made from (b) (4). The specifications for Tromethamine HCl are summarized in Table 99.

Table 99: Specifications for Tromethamine HCl (b) (4)

(b) (4)

3.2.P.4.2 and 3.2.P.4.3 Analytical Procedures and Validation of Analytical Procedures

The analytical procedures used for compendial excipients testing are performed according to the appropriate compendial monographs. The analytical procedures used to test Tromethamine HCl (b) (4) are summarized in Table 99. The assay methods are either compendial or validated except for the assays for (b) (4) and (b) (4) test is an

(b) (4) test and (b) (4) are not likely to be introduced during the manufacturing process.

3.2.P.4.4 Justification of Specifications for Excipients
The specifications of the compendial excipients are consistent with those required by the respective compendial monographs. The specifications of Tromethamine HCl are designed to confirm the identity and (b) (4)

3.2.P.4.5 Excipients of Human or Animal Origin
No excipients of human or animal origin are used in the DP.

3.2.P.4.6 Novel Excipient
There are no novel excipients in the DP.

Overall Reviewer's Assessment of Section 3.2.P.4:
- ❑ There are no concerns regarding the control of excipients used in the DP.

3.2.P.5 Control of Drug Product
3.2.P.5.1 and 3.2.P.5.6 Specification(s) and Justification of Specification(s)
Reviewed by EAG
The specification for SRP-9001 drug product release is summarized under the Table 100 *below. The justification for individual product attributes is summarized and discussed.*

Table 100: SRP-9001 Drug product release specification

Attribute	Analytical Procedure	Specification	Reviewer Comment
Appearance Clarity Color Visible particles Cap color	(b) (4) , visual inspection Visual inspection	Clear, colorless liquid, may have some opalescence, (b) (4) Cap color: Blue	*Acceptable, see discussion below. Amendment # 22 and 27*
(b) (4)	(b) (4)	(b) (4)	*Acceptable*
(b) (4)	(b) (4)	(b) (4)	*Acceptable*
Identity (vector genome)	(b) (4)	(b) (4)	*Acceptable*
Identity (vector capsid)	(b) (4)	(b) (4)	*Acceptable*

Sterility	(b) (4)	No growth	Acceptable
Bacterial Endotoxin	(b) (4)	(b) (4)	*Acceptable*
Capsid Purity	(b) (4)	(b) (4)	*Revise to* (b) (4) *(Amendment 22)*
(b) (4)	(b) (4)	(b) (4)	*Revise to* (b) (4) *under Amendment#22*
Percent Full Capsid	(b) (4)	(b) (4)	*Acceptable based on data and statistical analysis of process B lots*
Particulate Matter	Based on (b) (4)	(b) (4)	*Acceptable compendial limits*
(b) (4)	(b) (4)	(b) (4)	*Criterion based on* (b) (4)
Potency	(b) (4)	(b) (4)	*Applicant did not revise potency spec under Amendment 22. FDA recommend* (b) (4) *relative potency based study 103 DP lots with consideration for method variability*
		-- (b) (4) SRP-9001-micro-dystrophin: (b) (4)	*Attribute not stability indicating and can not detect degraded DP. Therefore acceptable.*
Vector Genome Concentration	(b) (4)	(b) (4)	*Revised based on IR response in Amendment 67 Acceptable see discussion*
Extractable volume	(b) (4)	(b) (4)	*Acceptable see DBSQC memo.*

Appearance: Release Acceptance Criterion: *Clear, colorless liquid, may have some opalescence,* (b) (4) *. Cap color: Blue*

Justification: Filled vials are manually inspected. Defective vials including those with particulates are rejected during 100% visual inspection release test as described in (b) (4)

5 pages determined to be not releasable: (b)(4)

Container Closure Integrity: Container closure integrity testing (CCIT) is used in lieu of sterility testing during the (b) (4) stability time points. A deterministic (b) (4) test is utilized as described in (b) (4) The acceptance criterion is based on the (b) (4) *See full detailed review under DMPQ memo. The method was found to be acceptable.*

(b) (4) **:** According to the applicant, this attribute is being replaced with the (b) (4) assay. *Note: The assay was used in the assessment of process A material with no specified limit. After the switch to process B, an acceptance criterion of* (b) (4) *. This criterion was not informative as it was still wide* (b) (4) *relative to actual manufacturing data. Note: this method has been discontinued under the commercial Process B.*

Overall Reviewer's Assessment of Sections 3.2.P.5.1 and 3.2.P.5.6:
- ❑ The release specification for the drug product and justifications are acceptable.

3.2.P.5.2 and 3.2.P.5.3 Analytical Procedures and Validation of Analytical Procedures
Reviewed by EAG

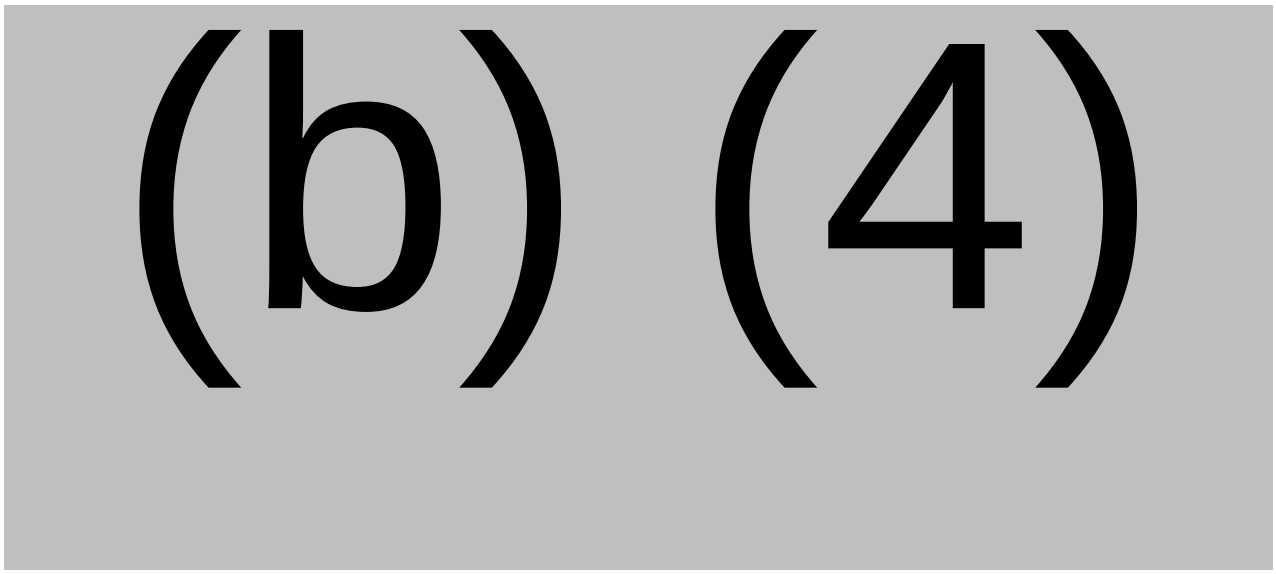
Compendial Analytical methods:
Note: For compendial methods such as (b) (4) *Appearance,* (b) (4) *, Sterility bacterial endotoxins, the description of the assay and verification information are reviewed in adequate details by DBSQC. Please see DBSQC review memo. The description and validation Product specific non-compendial analytical methods used for the release of SRP-9001 are summarized below.*
Product specific, non-compendial release methods have been discussed under DS section 3.2.S.4.2 Analytical Procedures and 3.2.S.4.3 Validation of Analytical Procedures*) for Identity (vector genome), Identity (Vector capsid, Protein Identity), Capsid Purity and* (b) (4)

- ❑ **Percent Full Capsids by** (b) (4)

(b) (4)

9 pages determined to be not releasable: (b)(4)

(b) (4)



(b) (4)

Extractable volume: *This compendial method was reviewed and found acceptable. See DBSQC memo for details.*

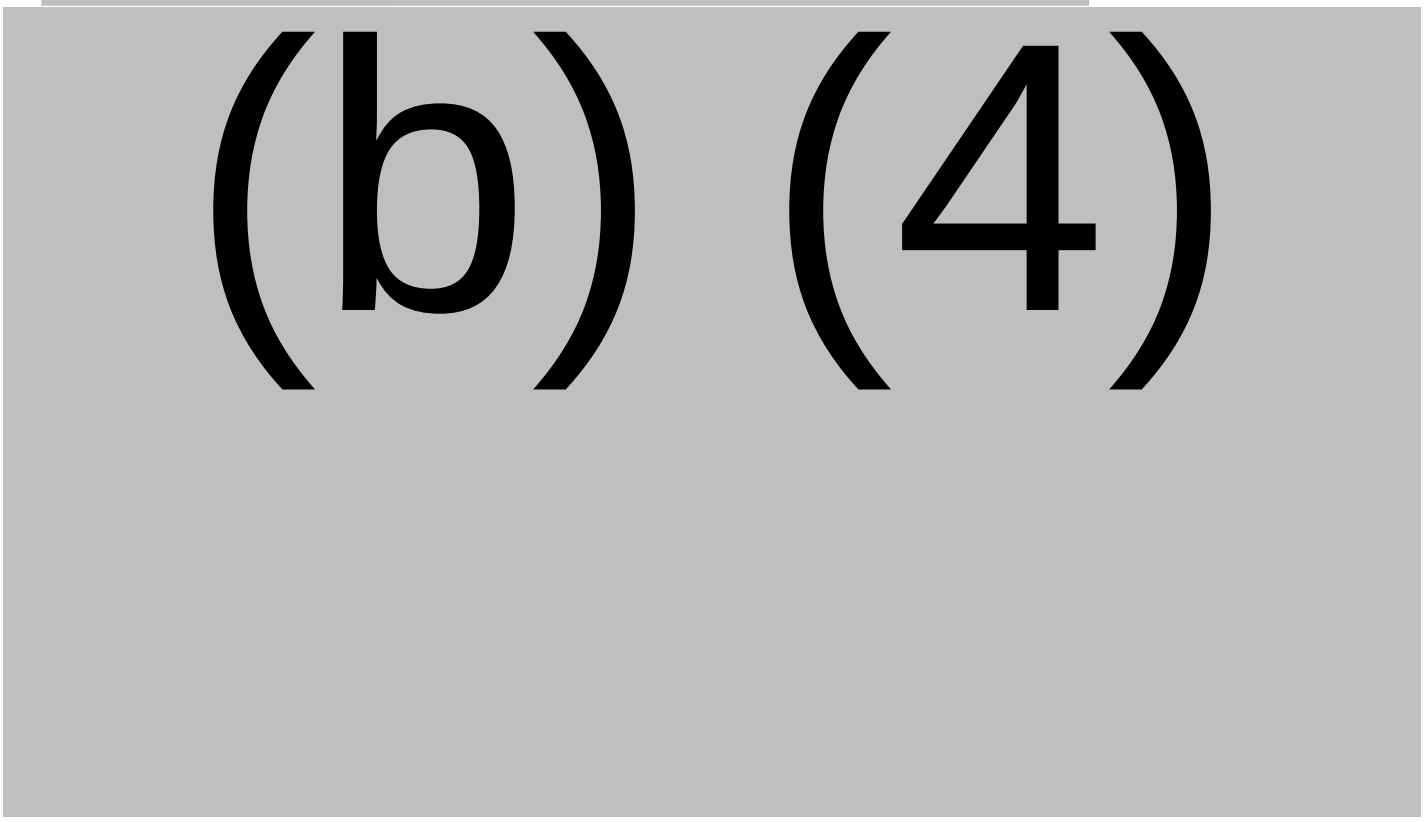
Overall Reviewer's Assessment of Sections 3.2.P.5.2 and 3.2.P.5.3:
The description of DP release methods and validation is acceptable.

(b) (4)

3.2.P.5.4 Batch Analyses

The information submitted to support batch analysis include process A and process B batches. Except for process B PPQ batches, all batches made were processed through to DP without a discrete DS storage stage. Because Process A has been discontinued and was unvalidated, the CMC review team's assessment of batch analysis was done primarily with process B batches which is the commercial representative process. Process A batches, used for Phase 1 Clinical trial(study101) have been reviewed and discussed under IND 17763. Drug product batch made from Catalent Process B process are summarized below.

(b) (4)



(b) (4)

(b) (4)

3.2.P.5.5 Characterization of Impurities

The DS and DP are identical except (b) (4) *DS batches are* (b) (4) *. The description of product and process-related impurities, their clearance and control was discussed under.* 3.2.S.3.2 Impurities*.*

Overall Reviewer's Assessment of Sections 3.2.P.5.4 and 3.2.P.5.5:
- ❑ Description of DP batch analysis and impurity and is acceptable.

3.2.P.6 Reference Standards or Materials

Refer to section 3.2.S.5 Reference Standards or Materials) for information on Reference Standards or Materials. Reference standard information is acceptable.

3.2.P.7 Container Closure System

Reviewed by EAG and AS

- o *Refer to information under section 3.2.P.2.4 Container Closure System.*

Overall Reviewer's Assessment of Section 3.2.P.7:
- ❑ Information submitted and the proposed PMC are acceptable.

3.2.P.8 Stability

Reviewed by SS

3.2.P.8.1 Stability Summary and Conclusion and 3.2.P.8.3 Stability Data

The intended storage condition for the DP is ≤ -60°C. DP stability studies include storage conditions of ≤ -60°C (up to (b) (4) months), (b) (4) (up to 6 months), and 2-8°C (up to 14 days), (b) (4) . The CCS used for DP stability studies is the same as the one used for commercial production. The proposed DP shelf life is 12 months at ≤ -60°C, and the DP is recommended to "Do not refreeze" and "Do not shake".

Data from the assays listed in the Table 112 were analyzed to determine the DP shelf life. The (b) (4) assay was replaced by the in (b) (4) potency assay during the clinical development and was discontinued. The Applicant proposed to revise the AC for DP stability studies in Amendment #19 (2/3/2023), Amendment #50 (5/3/2023), Amendment #66 (6/7/2023), and Amendment #70 (6/14/2023). The DP stability specification for clinical and PPQ batches and the revised stability specification submitted under Amendment #70 (6/14/2023) are summarized in the table below. The changes are shown in bold.

Table 112: DP Stability Acceptance Criteria

Attribute	Acceptance Criteria for Clinical and PPQ Batches	Acceptance Criteria for Commercial Batches
Appearance	Clear, colorless liquid, may have some opalescence, May contain white to off-white particles	Clear, colorless liquid, may have some opalescence, May contain white to off-white particles
(b) (4)	(b) (4)	(b) (4)
Vector Genome Concentration	(b) (4)	(b) (4)
Potency-(b) (4)	(b) (4)	(b) (4)
	(b) (4)	(b) (4)
(b) (4)	(b) (4)	N/A
Capsid Purity	(b) (4)	(b) (4)
(b) (4)	(b) (4)	(b) (4)
Particulate Matter Particles(b) (4) Particles (b) (4)	(b) (4)	(b) (4)

Bacterial Endotoxin	(b) (4)	(b) (4)
Sterility	No growth	No growth
Container Closure Integrity Test (CCIT)	(b) (4)	(b) (4)
* The (b) (4) assay was replaced by the in (b) (4) potency assay during the clinical development.		

Reviewer comment:

- *Vector genome titer: The Applicant proposed to revise the AC for vector genome titer for DP stability studies from (b) (4) (Amendment #19 dated 2/3/2023), to (b) (4) of the target titer) (Amendment #50 dated 5/3/2023), and then again to (b) (4) vg/mL (b) (4) (Amendment #66 dated 6/7/2023). Based on the stability data submitted, there are no changes in vector genome titer. FDA requested to revise the AC to be consistent with DP release, which is (b) (4) (6/8/2023). The Applicant accepted FDA recommendation and submitted the updated specification for the post-approval stability protocol under Amendment #70 (6/14/2023).*
- *The revised AC for (b) (4), potency (b) (4)), capsid purity, and (b) (4) have been submitted under Amendment #66 (6/7/2023). This is acceptable.*

DP stability at the long-term ≤ -60°C storage condition

The long-term stability study has been conducted using (b) (4) DP batches (Process B) as summarized in Table 113. The DP shelf-life was determined based on data collected from t(b) (4) registration DP batches. The Applicant provided updated stability data under Amendment #19 (2/3/2023).

Table 113: DP batches on long-term (≤ -60°C) stability studies

(b) (4)

Reviewer's comment:

- *In Amendment #47 submitted on 4/20/2023, the Applicant clarified that the DP vials used in the stability studies have not undergone the same labeling process as the proposed commercial DP at (b) (4). Although the Applicant submitted stability data*

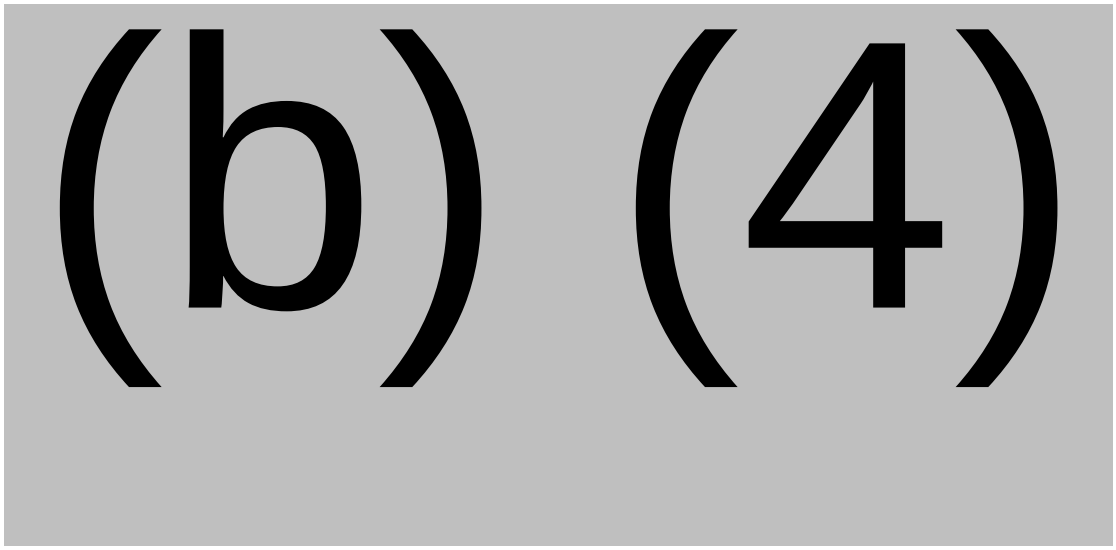
for the (b) (4) *study that mimics the labeling process, the current stability protocol does not represent the long-term storage condition of commercial DP vials. In Amendment #66 (6/7/2023), the Applicant agreed to include* (b) (4) *to mimic the commercial labeling and packaging process before the DP vials are stored at ≤ -60°C and submitted the updated post-approval protocol.*

- *The stability samples are shipped from the DP manufacturing site, Catalent BioPark, to* (b) (4)*, where they are* (b) (4) *. Upon receipt vials are placed in ≤ -60°C storage until tested.* (b) (4) *for CCIT and to Sarepta Andover for potency and vector genome titer. All other stability tests are performed at* (b) (4) *There are no concerns related to shipping and handling of the stability samples.*

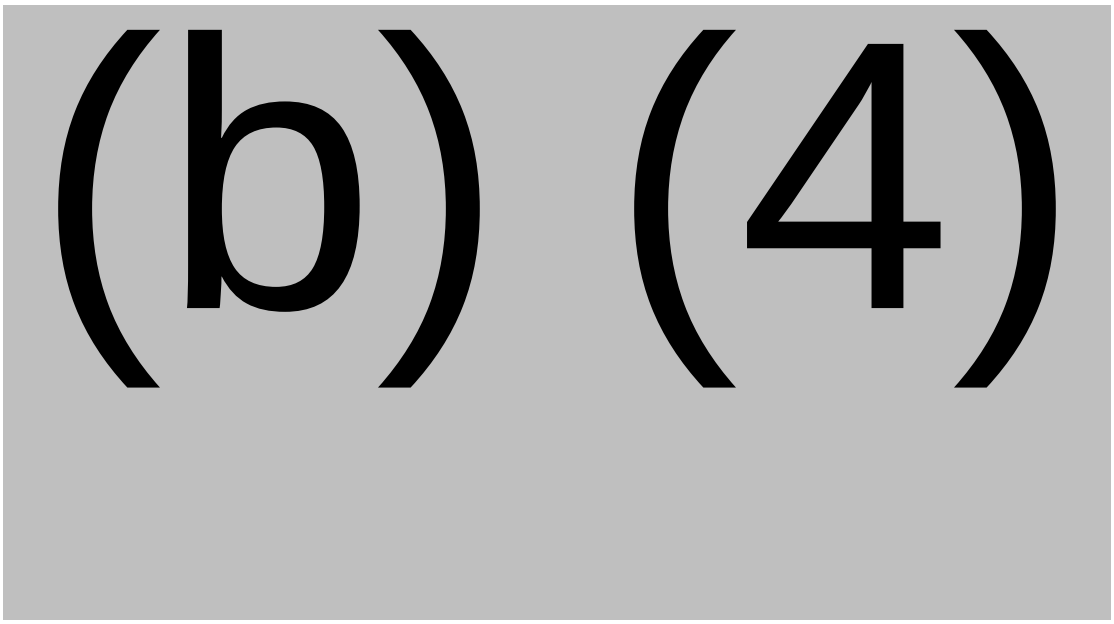
- Vector genome concentration

A plot of registration and PPQ stability data without a fit is shown in the figure below. The vector genome concentration results for the DP stored at ≤-60°C for up to (b) (4) months conform to the proposed AC.

Reviewer's comment: *Based on the data provided, no significant decreases in vector genome titer were observed. However, the assay results are highly variable, and a statistical evaluation of stability trends was not possible due to this high variation. Regarding* (b) (4) *assay re-validation, see Section 3.2.S.4.2 Analytical Procedures* and 3.2.S.4.3 Validation of Analytical Procedures.



- (b) (4)

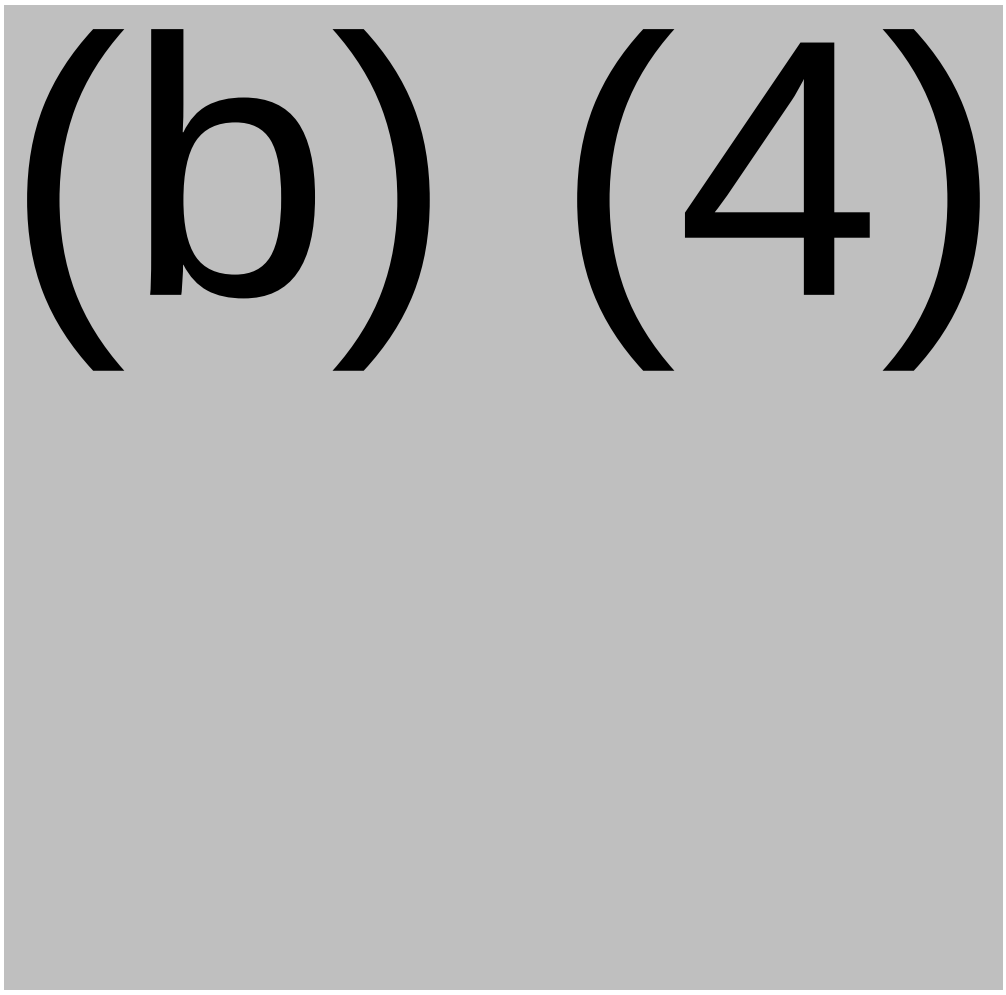


Reviewer comment:

- *The stability data for* (b) (4) *potency are highly variable. The Applicant stated that due to limited timepoints for each stability batch available (only* (b) (4) *stability timepoints for each lot), a statistical evaluation of stability data and stability trends using the available stability data is not possible (Amendment #47 dated 4/27/2023). Regarding assay controls and PMCs related to the potency assay, see* (b) (4) *Potency Assay.*
- *The AC for* (b) (4) *potency is* (b) (4) *and it is too wide to ensure the product stability. After several rounds of IRs, the Applicant agreed to revise the AC to* (b) (4) *in Amendment #66 (6/7/2023).*
- *During the PLI, it was discovered that the Applicant did not investigate multiple assay errors associated with* (b) (4) *potency testing on stability samples. This led to Observation 1 on the 483. See the EIR for the Sarepta Andover facility for additional information.*

- (b) (4) / Capsid purity

(b) (4) capsid highest impurity in all timepoints analyzed met the AC but showed a potential trend with time during storage at ≤-60°C. According to the Applicant, all prediction results were within the specification (b) (4) at (b) (4) months, based on the prediction model using the data from registration batches. The Applicant commits (b) (4) .



Stability at the accelerated storage condition (b) (4)

(b) (4)

Stability at the stressed condition (2-8°C)

The stability study for the stressed condition was conducted to support potential brief storage of the DP at 2-8°C prior to administration at the clinics. This study was conducted using (b) (4) Process B DP (b) (4) stored at 5 ± 3 °C condition in an upright orientation for 3, 7, and 14 days. Appearance, (b) (4) vector genome titer, (b) (4) , capsid purity, and (b) (4) were not changed up to 14 days. The count of particles larger than (b) (4) per container was increased from (b) (4) particles/container at T=0 to (b) (4) particles/container at 14-day, but all data points still met the proposed AC.

Reviewer's comment: *While the* (b) (4) *potency data for* (b) (4) *micro-dystrophin at 7-day and 14-day were unavailable due to operator errors, this potency assay is not stability indicating and the* (b) (4) *potency data measuring total micro-dystrophin at 7-day and 14-day*

met the AC. Therefore, it is acceptable to demonstrate that the DP is stable for up to 14 days when held at 5 ± 3 °C in the final DP container. However, the study was conducted using the DP vials held only in an upright configuration. FDA recommended to indicate that the DP is stable for up to 14 days at 5 ± 3 °C when the vial is held in an upright position in the package insert. A commitment to (b) (4) *is demonstrated in Section 3.2.P.8.2 Post-Approval Stability Protocol and Stability Commitment.*

(b) (4)

.

(b) (4)

(b) (4)

2 pages determined to be not releasable: (b)(4)

(b) (4)

(b) (4)

3.2.P.8.2 Post-Approval Stability Protocol and Stability Commitment

The Applicant commits to continuing and completing the ongoing stability studies for the registration lots to (b) (4) months at ≤-60°C storage conditions. The Applicant may extend the DP shelf life based on real-time data generated from three representative lots according to the ongoing stability study protocol. The shelf life will be updated in the annual report.

Annual stability

Annual stability studies will be performed on at (b) (4) at ≤-60°C and (b) (4) storage condition in alignment with ICH Q7. The post-approval stability protocol for the ≤-60°C storage condition is summarized in the table below. If no DP is manufactured during a given year, no stability study will be initiated. The Applicant also commits to placing a DP batch manufactured with major DS or DP manufacturing changes on stability.

Table 119: DP Stability Protocol for ≤-60°C Storage Condition

Test Name	Acceptance criteria	Time Point (month)					
		0	3	6	9	12	(b) (4)
Appearance	Clear, colorless liquid, May have some opalescence. May contain white to off white particles.	X	X	X	X	X	
Capsid Purity	(b) (4)	X	X	X	X	X	
Vector Genome Concentration	(b) (4)	X	X	X	X	X	

(b) (4)	(b) (4)	X	X	X	X	X	(b) (4)
(b) (4)	(b) (4)	X	X	X	X	X	
(b) (4) potency	(b) (4)	X	X	X	X	X	
	(b) (4)	X	X	X	X	X	
Particulate Matter Particles (b) (4) Particles (b) (4)	(b) (4) (b) (4)	X	--	--	--	X	
Endotoxin	(b) (4)	X	--	--	--	X	
Sterility	No grow	X	--	--	--	--	
Container Closure Integrity Testing	(b) (4)	--	--	--	--	X	
-- Not tested							

In-use stability

The in-use DP compatibility study was performed on DP lots less than 12 months old at the time of the study provided in Section 3.2.P.2.6 Compatibility. The Applicant commits to (b) (4)

Stressed (5±3°C) stability

The stressed stability study was performed on (b) (4) potency results at 7-day and 14-day were invalidated due to operator error. The Applicant commits (b) (4)

Reviewer's comment: *The Applicant agreed to include (b) (4) to mimic the commercial labeling and packaging process and submitted the updated post-approval protocol under Amendment #66 (6/7/2023). Applicant's post-approval stability plan and stability commitments are acceptable.*

Overall Reviewer's Assessment of Section 3.2.P.8:
(b) (4) registration lots and (b) (4) PPQ lots were evaluated for DP stability. After multiple rounds of IRs, the Applicant agreed to revise the stability AC for vector genome titer to be consistent with DP release, which is (b) (4) and submitted the updated post-approval stability protocol under Amendment #70 (6/14/2023). The revised AC for (b) (4) potency (b) (4) capsid purity, (b) (4) were also submitted under Amendment #66 (6/7/2023). The stability data for (b) (4) potency are highly variable, and a statistical evaluation of stability data and stability trends using the available stability data is unavailable due to limited timepoints for each stability batch (only (b) (4) stability timepoints for each lot). FDA recommended that the Applicant tighten the AC for in (b) (4) potency (b) (4), and the Applicant agreed to revise the AC to (b) (4) and submitted the updated post-approval protocol in Amendment #66 (6/7/2023).

The DP vials used in the stability studies have not undergone the same labeling process as the proposed commercial DP, which does not represent the long-term storage condition of commercial DP vials. The Applicant agreed to include (b) (4) to mimic the commercial labeling and packaging process before the DP vials are stored at ≤ -60°C and submitted the updated post-approval protocol under Amendment #66 (6/7/2023).

Based on data from (b) (4) registration lots, the Applicant proposed a 12-month shelf life for the DP at ≤-60°C. Additional data from the ongoing stability study will be provided as it becomes available in order to extend the DP shelf life. This is acceptable.

The stability data for the stressed condition were provided to support potential brief storage of the DP at 2-8°C and room temperature (25°C) prior to administration at the clinics. The results support that the DP is stable for up to 14 days when held at 5 ± 3 °C and stable for up to 24 hours at room temperature (25°C). However, the studies were conducted using the DP vials held only in an upright configuration. Following FDA's recommendation, the package insert was revised to indicate the position of DP vials. In addition, (b) (4), and (b) (4) studies were performed appropriately.

3.2.A APPENDICES

3.2.A.1 Facilities and Equipment

Reviewed by DMPQ

3.2.A.2 Adventitious Agents Safety Evaluation

Reviewed by SS

The strategy to control adventitious agent comprises of:

1. Ensuring adequate control of raw materials, especially those of biological origin that are used in the generation of cell banks and DS manufacturing.
2. Testing of cell banks and unprocessed bulk harvest for adventitious agents (bacteria, fungi, mycoplasma, and viruses)
3. Viral clearance by spike-recovery studies using (b) (4) model viruses to demonstrate that the downstream purification process can effectively clear viruses exhibiting a broad range of biochemical and biophysical properties.

Reviewer's comment: *Materials of Biological Origin including cell banks, (b) (4) were reviewed in 3.2.S.2.3 Control of Materials. The materials are satisfactorily controlled.*

- **Viral Clearance Studies**

Reviewed by LB

(b) (4)

(b) (4)

(b) (4)

Reviewer's Assessment:

The viral clearance study results are acceptable.

Overall Reviewer's Assessment of Section 3.2.A.2:

- The information provided in Module 3.2.A.2 demonstrates negligible risk posed by materials of biological origin and demonstrates robust viral clearance. This is acceptable.

3.2.A.3 Novel Excipients

There are no novel excipients.

3.2.R Regional Information (USA)

- **Executed Batch Records**

Reviewed by EAG and LB

A representative set of executed DS upstream and downstream batch records are provided for all steps in the DS manufacturing process described (under 3.2.S.2.2 Description of Manufacturing Process). The batch record for DS PV/PPQ DS batch (b) (4) was submitted in the BLA. The batch record contained detailed step-by-step executed instruction and data of manufacturing activities of all the steps involved in DS manufacture; from (b) (4) for SRP-9001.

A representative batch record for the preparation of the formulation buffer used during DS and DP manufacture (Lot No. (b) (4)) was also submitted and reviewed to be adequate. Additionally, a copy of an executed batch record for the DP detailing the manufacture of DP PV/PPQ batch # (b) (4) was submitted and reviewed to be generally acceptable.

Reviewer Note: *During pre-license inspection (PLI) at the DS site (March 6-10, 2023, BWI-Harman Maryland). I(EAG) reviewed additional post PPQ executed batch records for DS Lot#*(b) (4) *and Lot#*(b) (4)*. Also, during PLI for the DP (Feb 24-27, Biopark -Baltimore Maryland), additional executed batch records for DP lots* (b) (4) *and formulation buffer lots* (b) (4) *were reviewed. No concerns were found. The executed batch records for SRP-9001 DS and DP are acceptable.*

Overall Reviewer's Assessment of Combination Products Section:
- ❑ Executed batch records submitted for review are acceptable.

- ❑ **Method Validation Package**

Method validation package provided in the BLA was reviewed and discussed at the appropriate sections of this memo: 3.2.S.4.2 Analytical Procedures and 3.2.S.4.3 Validation of Analytical Procedures *(for drug substance) and 3.2.P.5.2 and 3.2.P.5.3* Analytical Procedures and Validation of Analytical Procedures *(for Drug Product).*

- ❑ **Combination Products**

Not applicable

- ❑ **Comparability Protocols**

This is not applicable to this BLA. The Sponsor did not propose a future comparability study.

Other eCTD Modules

Module 1

A. Environmental Assessment or Claim of Categorical Exclusion

Reviewed by EAG

The Applicant submitted environmental (EA) assessment under section 1.12.14 of the BLA, in accordance with 21 CFR 25 requirement. The applicant does not make a claim of categorical exclusion for EA. This application is not eligible for categorical exclusion.

The product delandistrogene moxeparvovec-rokl is derived from rhesus serotype 74 [rh74]) AAV (AAVrh74), a nonpathogenic human DNA virus that is incapable of self-replication. The natural DNA genome of AAVrh74 has been replaced with SRP-9001 DNA for the expression of the miniaturized dystrophin protein. Vector mobilization theoretically may occur in the rare setting of a helper virus infected patient because of complementation and recombination between the viral vector, WT AAV and a helper virus. A worst-case scenario (presence of all helper functions plus AAV vector) would yield very low levels of additional vector, which is non-pathogenic. The replication of recombinant AAVrh74 in an infected host cell is dependent on co-infection with a WT AAV virus and a helper virus such as adenovirus. The generation time of Wt AAV in a natural ecosystem will be significantly very high, depending on the timing of the coinfection. The generation of replication competent AAV (rcAAV) at the time of SRP-9001 is not relevant since it lacks the rep and cap genes that are required for replication.

The manufacturing process is designed to minimize the potential that DNA recombination might result in a virus that contains viral DNA. The product is tested for the presence of (b) (4)

Certain wild-type AAVs can integrate at a specific locus of the host cell genome (AAVS1 in human chromosome 19 long arm); in these cases of integration, they remain non-pathogenic. The oncogenicity due to integration and insertional mutagenesis is a potential risk of AAV vectors, based on findings of tumors in mice and hepatocyte clonal expansion in the livers of hemophilic dogs years after administration of an AAV vector, with insertions noted near genes that control cell growth (Nguyen 2021). In contrast, recombinant AAVs have lost the ability to integrate at specific sites in the host cells. Theoretical insertional mutagenesis, caused by non-site-specific integration of the SRP-9001 genome into the host cell genome, can occur in transfected cells. Also, the simian virus 40 [SV40] sequence) present in the construct may, in theory, allow interaction of SRP-9001 viral sequences with viruses present in the patient or non-target individual, the lack of intact MHCK7 in other WT viruses makes recombination/mutagenesis a theoretical safety concern.

Germline transmission was evaluated in 2 nonclinical studies in DMD^{MDX} and WT mice, respectively per study #SR-20-014. Analysis of testes and ovaries using (b) (4) assay (b) (4) showed no staining above the negative control background for the AAV vector MHCK7 or the SRP-9001 transgene. SRP-9001 is indicated for use in male children. the applicant concludes that vertical transmission, via germline transmission is negligible.

Shedding occurs through patient excreta. Caregivers and patient's families will be advised on the proper handling of patients' bodily fluids and waste. Standard precautions are recommended to the health care providers, including the pharmacy personnel preparing SRP-9001, and waste should be disposed of as regulated medical waste. For caregivers, standard hygiene measures are recommended for caregivers and treated subjects after SRP-9001 treatment.

Data from a clinical study demonstrate that patients who are treated with delandistrogene moxeparvovec will shed vector DNA in saliva, urine, feces for around 4 wks. DNA will also be shed in semen for extended period of time after administration. It is not known how much of the shed DNA is encapsidated in AAV capsids, as opposed to shedding of naked DNA. Even if encapsidated, the risk of causing infectious disease is zero because the product is inherently incapable of causing infectious disease, and there will be no direct toxic effects from exposure to small amounts of this vector, even if it is intact. The likelihood of germline transmission of vector DNA through semen is negligible. Animal studies showed no indication of paternal germline transmission to the offspring, even with high levels of vector DNA present in gonads. Please refer to pharmacology/toxicology memo for additional details. The AAV vector DNA in the semen is mainly present in the seminal fluid and not in the sperm cells, which is necessary for the germline transmission to the host progeny genome.

This product will be administered at hospitals or treatment centers using universal precautions, and unused product and product-contact materials will be disposed of as biohazardous medical waste. The product is relatively stable (compared to other viruses) at room temperature but will degrade over time into naturally occurring materials. Data from a clinical study demonstrate that patients who are treated with delandistrogene moxeparvovec-rokl will shed vector DNA in saliva, urine, feces for around 4-weeks. Viral shedding peaks in the first 48 hours post SRP-9001 administration in saliva and urine) and first month in the feces, then decreases rapidly to a level below the LOD. The half-lives (mean range) are ~57 to ~68 hours in saliva, ~38 to ~45 hours in urine, and~54 to ~57 hours in feces. The data from the viral shedding assessment in clinical patients also show decrease in shedding from peak to week 4 was greater than 99% for saliva, urine, and feces.

Reviewer Comment:

- *The information provided in the environmental assessment demonstrate that the SRP-9001 poses no significant environmental risk from its approval. As such, a finding of no significant impact (FONSI) will be prepared.*

B. (b) (4)

C. Labeling Review
Full Prescribing Information (PI):
Reviewed by EAG

Sections 2 (Dose and Administration) and 3 (Dosage Forms and Strengths)

ELEVIDYS is supplied as a frozen suspension of adeno-associated virus (AAV) vector-based gene therapy for a single intravenous infusion with a nominal concentration of 1.33×10^{13} vg/mL. It is supplied to the clinic as a customized commercial kit containing ten(10) to seventy (70) 10 mL single-dose vials. Each kit constitutes a dosage unit based on the patient's body weight. The individual product vial and each of the possible kits has a separate NDC number. The recommended dose of the product is 1.33×10^{14} vector genomes per kilogram (vg/kg) of body weight (or 10 mL/kg body weight) and it is administered as a single intravenous infusion without dilution at a rate of less than 10 mL/kg/hour.

Prior to administration, the number of single-dose vials and volume of product needed (based on patient weight) is calculated and verified. The dose needed is transferred into the recommended syringe using aseptic techniques. (Multiple syringes maybe be prepared depending on the patient weight). The dose is administered via a syringe infusion pump, IV infusion tubing and catheter equipped with a 0.2-micron PES in-line filter. The intravenous access line is flushed with 0.9% Sodium Chloride Injection before and after the infusion.

Section 11 (Description)
ELEVIDYS (delandistrogene moxeparvovec-rokl) is a recombinant gene therapy designed to deliver the gene encoding the ELEVIDYS micro-dystrophin protein. ELEVIDYS is a non-replicating, recombinant, adeno-associated virus serotype rh74 (AAVrh74) based vector containing the ELEVIDYS micro-dystrophin transgene under the control of the MHCK7 promoter. The micro-dystrophin protein expressed by ELEVIDYS is a shortened version of dystrophin (138 kDa, compared to 427 kDa size of dystrophin expressed in normal muscle cells) that contains selected domains of dystrophin expressed in normal muscle cells.

ELEVIDYS is a preservative-free, sterile, clear, colorless liquid that may have some opalescence and may contain white to off-white particles. ELEVIDYS is a suspension for intravenous infusion with a nominal concentration of 1.33×10^{13} vg/mL and supplied in a single-dose 10 mL vial. Each vial contains an extractable volume of 10 mL and the following excipients: 200mM sodium chloride, 13 mM tromethamine HCl, 7 mM tromethamine, 1mM magnesium chloride, 0.001% poloxamer 188.

Section 12 (Clinical Pharmacology)

ELEVIDYS is designed to include MHCK7 promoter/enhancer that drives transgene expression in skeletal muscle cells. In nonclinical studies, ELEVIDYS micro-dystrophin protein was expressed predominantly in skeletal muscle (including diaphragm) and cardiac muscle cells. In clinical studies, muscle biopsy analyses confirmed ELEVIDYS micro-dystrophin expression in skeletal muscle of patients. ***Note:*** *This section of the PI also contains adequate description of the biodistribution of the vector including the shedding of the virus after receiving the product. The assays used in the shedding, animal and human biodistribution studies are reviewed below in the sections for module 4/5.*

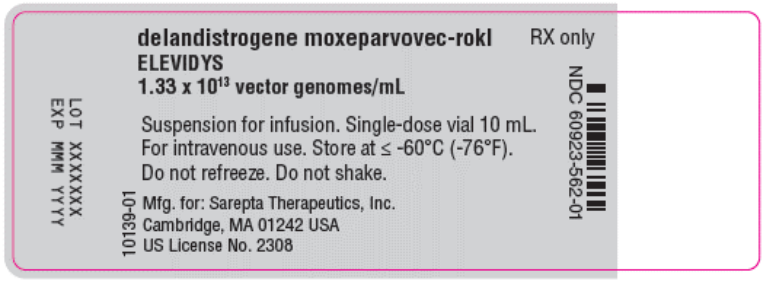
Section 16 (How supplied / storage and handling)
ELEVIDYS is shipped frozen (≤ -60ºC [-76ºF]) in 10 mL single-dose vials. It can be refrigerated for up to 14 days when stored at 2°C to 8°C (36º F to 46º F) in the upright position. It is supplied as a customized kit to meet dosing requirements for each patient. Each kit contains ten (10) to seventy (70) single-dose vials of ELEVIDYS and one alcohol wipe per vial. Each ELEVIDYS kit may contain a maximum of two different drug product lots.

Reviewer Note: *If vials from two different lots are kitted, the expiry is assigned based on the lot with the shortest shelf-life. The instructions provided in the PI is supported by the information submitted and reviewed in the BLA. This includes stability and storage conditions prior to and during use in the clinic*

Carton and Container Label:
Reviewed by EAG

After labeling, the primary vials are kitted(packaged) into a carton. The carton size ranges from 10 vial-carton to 70 vial-carton. Each kit bears unique NDC code#. Individual vial labels have NDC codes (and nominal titer). See below.

Figure 35: SRP-9001 Container/vial sample label



Reviewer Comment:
The primary vial and carton sample labels were reviewed and found acceptable per the requirements under 21 CFR Sec. 610.60-63 Container label. The Applicant under Amendment #70 (2023.06.14) revised the term 'single-use' vial to 'single-dose' vial to reflect the current FDA guidance (https://www.fda.gov/media/117883/download) and also included the language 'Do not shake' to the vial and carton labels. See additional schematic details about the kit configuration and Carton sample label under Figure 36*,* Figure 37 *and* Figure 38*. Finally, the Applicant provided a sample of the carton printed label that will be attached to the configured Kit (Amendment # 77, dated 2023.06.21) which included an updated suffix (*Figure 39*). This is acceptable.*

An example of carton(kit) label which will include a printed carton label (specifying the number of vials in a specific configured kit, product identifying information) are shown below in Figure 38 *and* Figure 39*.*

Figure 36: SRP-9001 One pack carton sample label

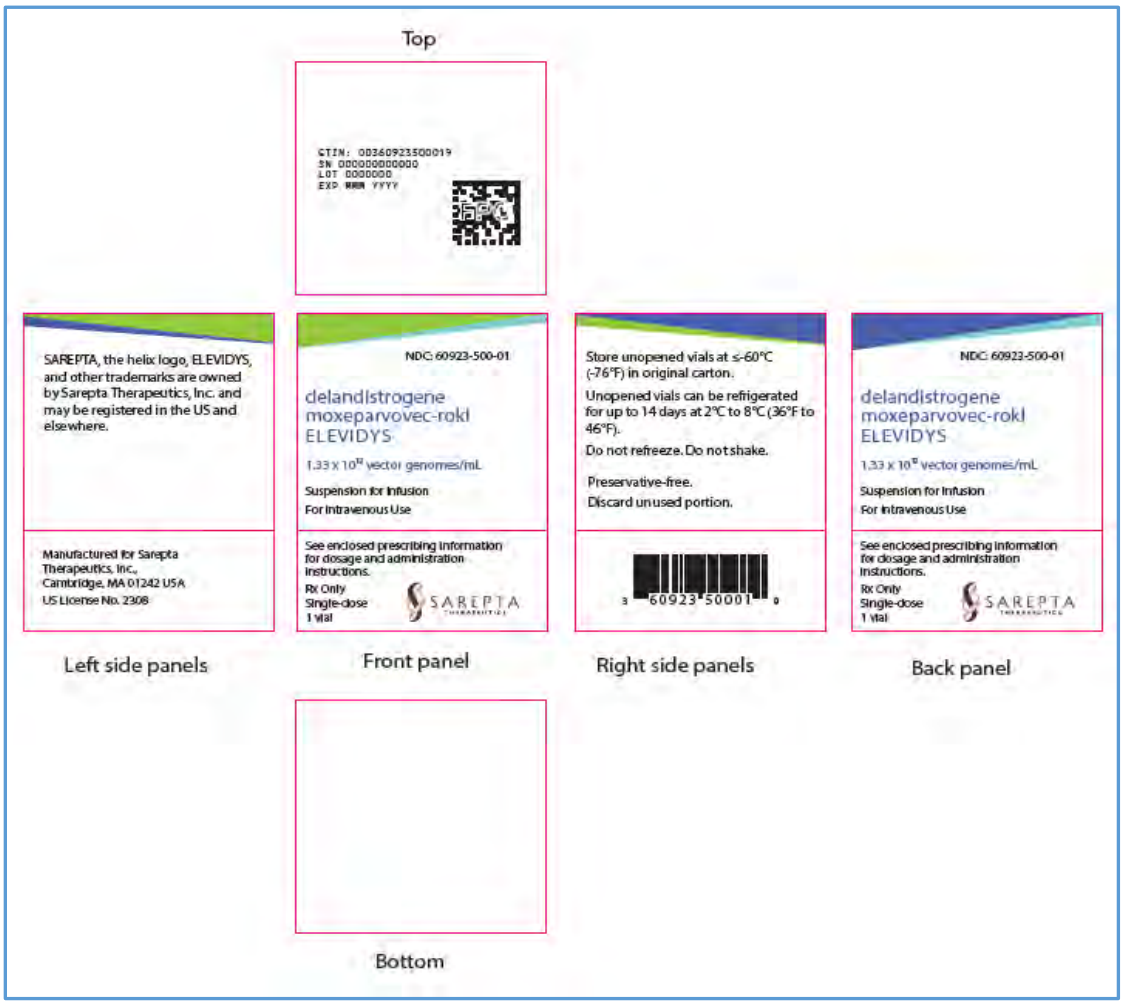


Figure 37: Example of Carton (kit) configuration

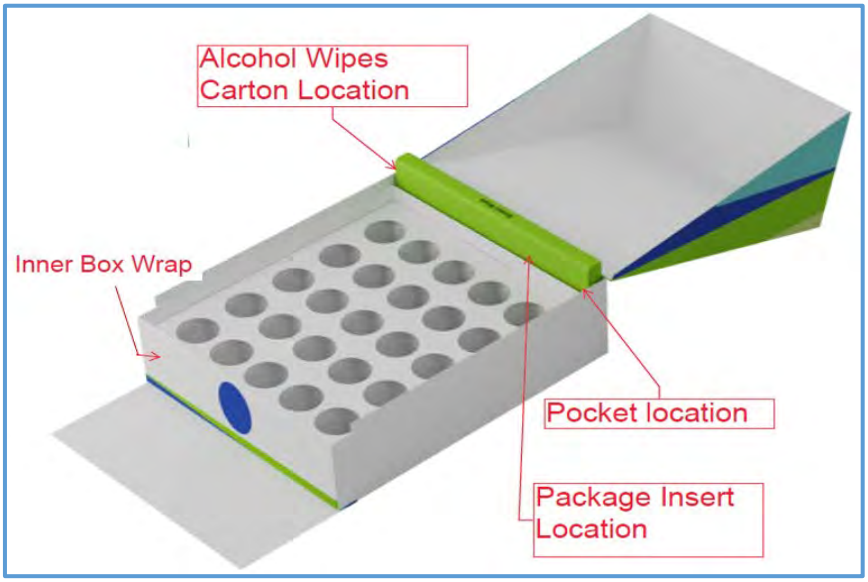
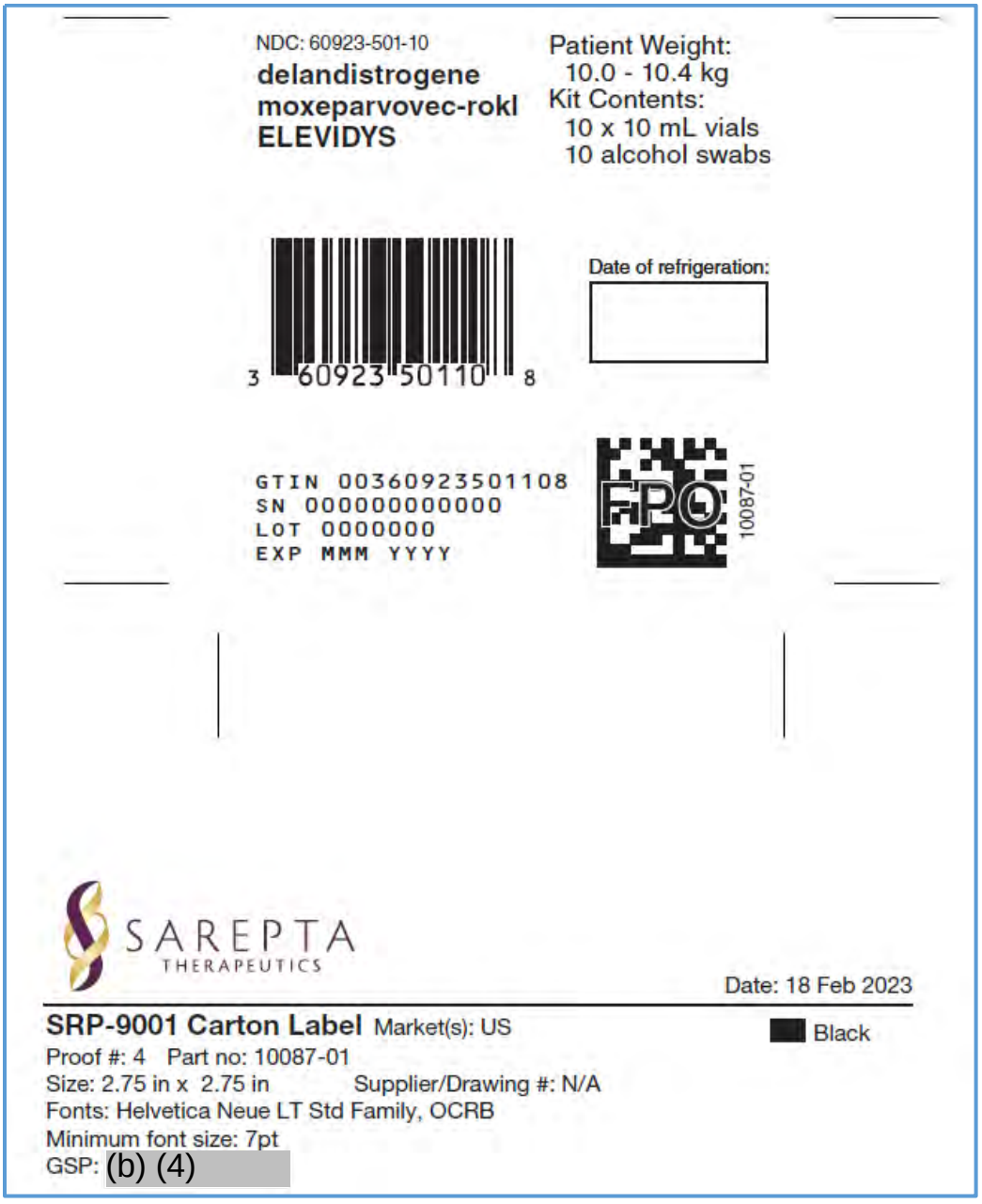


Figure 38: Example of Carton (Kit) front label

Figure 39: Printed ELEVIDYS Kit label



Modules 4 and 5

Analytical Procedures and Validation of Analytical Procedures for Assessment of Clinical and Animal Study Endpoints

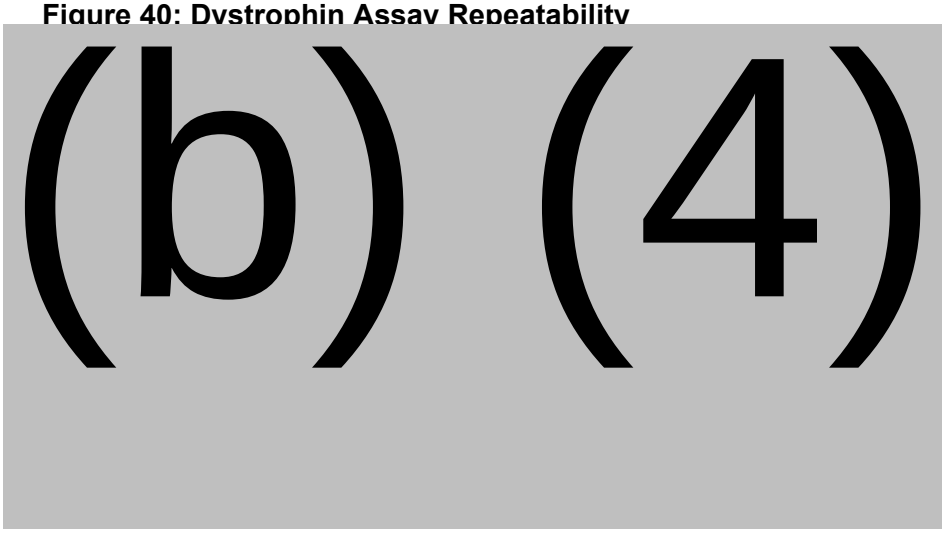
Fluorescent Immunohistochemistry Assays

Reviewed by Brian Stultz (BS)

The Applicant, in coordination with (b) (4), has developed fluorescent immunohistochemistry assays to monitor changes to the dystrophin associated protein complex (DAPC), dystrophin expression, and muscle content between pre- and post-treatment muscle biopsies as a biomarker for SRP-9001 efficacy. The Applicant has established a (b) (4)

(b) (4) fluorescent immunohistochemistry assay including muscle sectioning, antibody staining, image acquisition, image handling, and analysis algorithms to generate data. Antibodies are validated and fit for purpose for each assay. Muscle section antibody staining, and imaging protocols are optimized and validated. Imaging and analysis are mostly automated to ensure consistency across all samples with pathologist verification. Overall, the assay methodology is suitable to produce reliable data on comparison between pre- and post-treatment muscle biopsies. An example of assay performance for inter-run precision (intra-class correlation coefficient = (b) (4) is provided in the figure below.

Figure 40: Dystrophin Assay Repeatability



(b) (4) independent pathologist and analyst teams annotated and analyzed (b) (4) samples labeled on (b) (4) different days. The scores are highly clustered for each sample supporting the conclusion that myofiber MSD was not impacted by different days, different pathologists, or analyst teams.

Microdystrophin Western Blot Assay (Method v2, DOC-03106)

Reviewed by EAG

This method measures the absolute amount of SRP-9001 expressed microdystrophin via western blot. The method is performed in Andover and was performed to support clinical trial testing in the Phase I/II Clinical Trial for Duchenne Muscular Dystrophy using SRP-9001 (NCT03375164).

Table 121: Summary of Clinical Western Blot Assay description

Bioanalytical method	VAL-RPT-01333 Translational Development Microdystrophin Western Blot Method Version-2
(b) (4)	(b) (4)

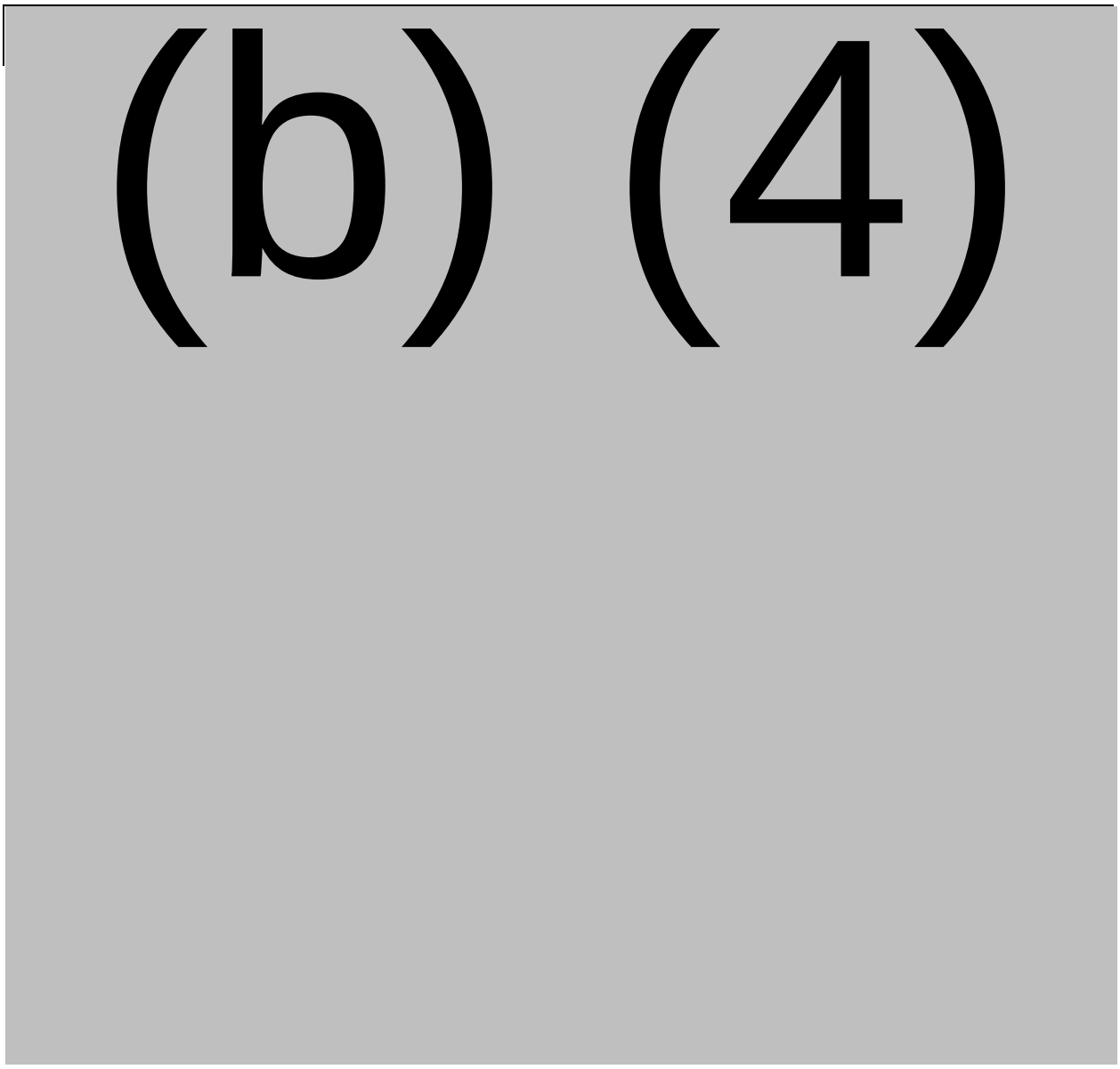
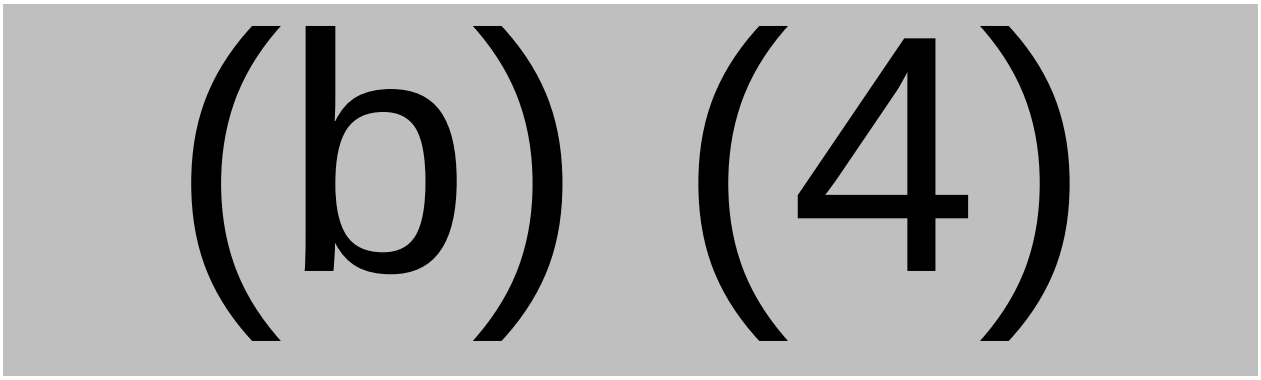


Table 122: Summary of Clinical Western Blot Assay Validation



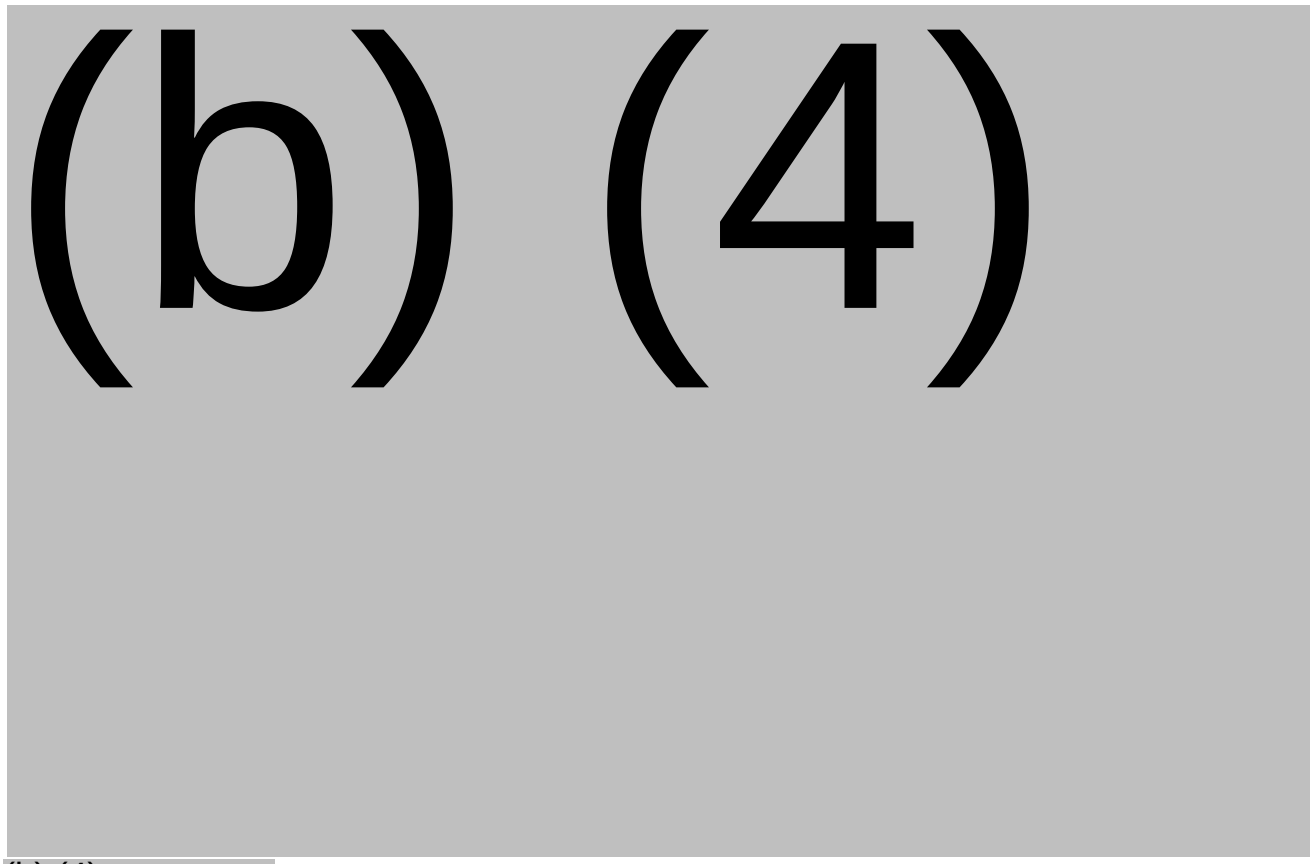
1 page determined to be not releasable: (b)(4)

Western blot assay will not be used as primary endpoint or for clinicals decision in the ongoing Phase 3 confirmatory trial.

Summary Method Performance for Biodistribution VGC ddPCR (DOC-03188)

DOC-02886:	Muscle DNA Isolation Method – for Muscle Tissue for ddPCR

(b) (4)



(b) (4)

Vector Shedding (Vector Genome Copies Assay): Analysis performed to measure the levels of vector genome copies shed into human stool, urine and saliva were conducted per the following:

- Vector Shedding (b) (4) (Vector Shedding in Stool method-SOP-DOC-(b) (4)
- Vector Shedding (b) (4) (Vector shedding in Urine and Saliva- SOP-DOC-(b) (4)

Briefly, the (b) (4) assay detects the viral genome DNA in a single reaction. The assay detects and quantify the absolute copy numbers of the viral genome present in certain amount of saliva, urine, and stool collected from patients who have received the SRP-9001 PRODUCT. The reaction relies on a (b) (4)

Critical reagents used:

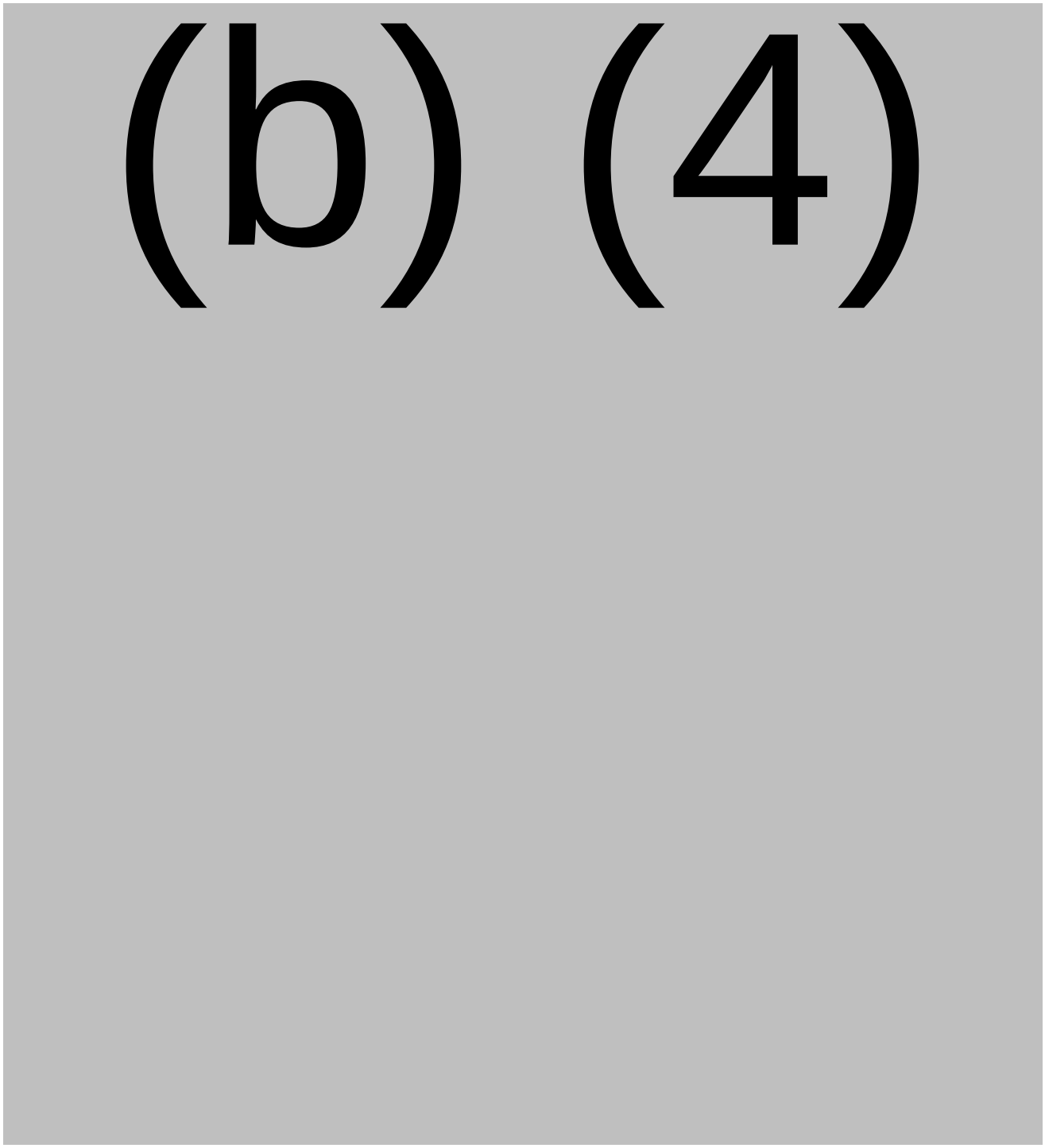
- (b) (4)

- (b) (4)

The extraction of vector DNA (using sample specific commercial kits) and the (b) (4) reaction are described appropriately under the following documents submitted to the BLA:

Table 123: Viral Shedding method summary and Validation

(b) (4)



AAVrh74 humoral immune response by ELISA

Reviewed and documented Dr Natasha Thorne, DIHD/OHT7(OIR)/OPEQ/CDRH.

- srp-9001-doc-02845.pdf – a “Test Method”/assay protocol for an ELISA Assay conducted at “the Gene Therapy Center of Excellence (GTCOE), at Sarepta, OH.”
- srp-9001-doc-02867.pdf – a protocol for the validation studies “to demonstrate the validity” of the ELISA Assay conducted at “the Sarepta Gene Therapy laboratory located at 5200 Blazer Parkway, Building 4, Dublin, OH 43017.”

- srp-9001-doc-02992.pdf – the results from the validation studies described in the srp-9001-doc-02867 document for the ELISA Assay as conducted at "the Sarepta Gene Therapy Center of Excellence (GTCOE) laboratory located at 5200 Blazer Parkway, Building 4, Dublin, OH 43017."

Excerpts from Consults Review: She concludes that:

- *there is insufficient information to fully understand the validation studies conducted*
- *The device as described in the "Test Method" document is different than the device evaluated in these method validation studies,*
- *samples are diluted at* (b) (4) *, that there is no evaluation of performance of the assay at higher dilutions* (b) (4) *or greater). The Applicant set the screening cut off at 1:400 or greater which is not supported by the validated cut off point.*
- *CDRH concludes that based on the information provided it is unable to determine the reliability of the assay.*

Overall Reviewer's Assessment of Relevant Sections of Module 4 and 5:

- ❑ Description and validation of assays for the assessment of clinical surrogate endpoint (WB assessment od microdystrophin) and viral shedding are acceptable.
- ❑ The ELISA assay for screening patients who will receive the drug is not adequate per consults review from Dr Natasha Thorne. The Applicant has submitted a PMR for consideration by the clinical review team. Defer review to clinical team.

Exhibit H

Catalent BIOLOGICS

Contact Us

Sarepta and Catalent Expand Strategic Manufacturing Partnership with Commercial Supply Agreement for Duchenne Muscular Dystrophy Gene Therapy Candidate

SOMERSET, N.J. and CAMBRIDGE, Mass. – January 5, 2023 — Catalent, Inc. (NYSE:CTLT), the leader in enabling the development and supply of better treatments for patients worldwide, and Sarepta Therapeutics, Inc. (NASDAQ:SRPT), the leader in precision genetic medicine for rare diseases, today announced the signing of a commercial supply agreement for Catalent to manufacture delandistrogene moxeparvovec (SRP-9001), Sarepta's most advanced gene therapy candidate for the treatment of Duchenne muscular dystrophy (DMD). The agreement also structures how Catalent may support multiple gene therapy candidates in Sarepta's pipeline for limb-girdle muscular dystrophy (LGMD).

In November 2022, Sarepta announced that the U.S. Food and Drug Administration (FDA) had accepted its biologics license application (BLA) seeking accelerated approval of delandistrogene moxeparvovec. Under the terms of this expanded

agreement, Catalent will be Sarepta's primary commercial manufacturing partner for this therapy.

"Sarepta is working as quickly as possible to advance new genetic medicines to treat progressive neuromuscular diseases like Duchenne and LGMD. We are excited to strengthen and expand our relationship with Catalent to meet anticipated demand for SRP-9001 and develop commercially scalable processes for additional gene therapy programs in our pipeline," said Doug Ingram, Sarepta's President and Chief Executive Officer. "We appreciate the years of dedication and collaboration that Catalent has provided in supporting our clinical trials for SRP-9001, and we look forward to continuing our work together through this expanded partnership."

"Our partnership with the Sarepta team spans nearly a decade across multiple programs and modalities, and we look forward to working together to manufacture these potentially life-changing and life-saving products for patients diagnosed with DMD and LGMD," said Alessandro Maselli, Catalent's President and Chief Executive Officer. "We look forward to leveraging our deep expertise in gene therapy development, manufacturing, and commercialization to support these programs as they advance toward potential regulatory approval."

Catalent's gene therapy network includes state-of-the-art facilities that currently house 10 cGMP gene therapy manufacturing suites, with another 8 suites under construction, each capable of accommodating multiple bioreactors up to 2,000-liter scale. For gene therapy development, customers can leverage the company's UpTempo Virtuoso℠ adeno-associated virus (AAV) platform, a scalable, GMP-ready process for viral vector manufacturing that can reduce a typical 18-month development timeline for drug product by half. Catalent is also the only contract development and manufacturing organization (CDMO) with a facility approved by the FDA for commercial manufacturing of an AAV gene therapy.

NOTES FOR EDITORS

ABOUT CATALENT

Catalent is the global leader in enabling pharma, biotech, and consumer health partners to optimize product development, launch, and full life-cycle supply for patients around the world. With broad and deep scale and expertise in development sciences, delivery technologies, and multi-modality manufacturing, Catalent is a preferred industry partner for personalized medicines, consumer health brand extensions, and blockbuster drugs.

Catalent helps accelerate over 1,000 partner programs and launch over 150 new products every year. Its flexible manufacturing platforms at over 50 global sites supply around 80 billion doses of nearly 8,000 products annually. Catalent's expert workforce of approximately 18,000 includes more than 3,000 scientists and technicians.

Headquartered in Somerset, New Jersey, the company generated nearly $5 billion in revenue in its 2022 fiscal year. For more information www.catalent.com.

MORE PRODUCTS. BETTER TREATMENTS. RELIABLY SUPPLIED.™

ABOUT SAREPTA THERAPEUTICS

Sarepta is on an urgent mission: engineer precision genetic medicine for rare diseases that devastate lives and cut futures short. We hold leadership positions in Duchenne muscular dystrophy (DMD) and limb-girdle muscular dystrophies (LGMDs), and we currently have more than 40 programs in various stages of development. Our vast pipeline is driven by our multi-platform Precision Genetic Medicine Engine in gene therapy, RNA and gene editing. For more information, please visit www.sarepta.com or follow us on Twitter, LinkedIn, Instagram and Facebook.

CATALENT FORWARD LOOKING STATEMENTS

This release contains both historical and forward-looking statements. All statements other than statements of historical fact, are, or may be deemed to be, forward-looking statements within the meaning of Section 27A of the Securities Act of 1933, as amended, and Section 21E of the Securities Exchange Act of 1934, as amended. These forward-looking statements generally can be identified by the use of statements that include phrases such as "believe," "expect," "anticipate," "intend," "estimate," "plan," "project," "foresee," "likely," "may," "will," "would," or other words or phrases with similar meanings. Similarly, statements that describe Catalent's objectives, plans, or goals are, or may be, forward-looking statements. These statements are based on current expectations of future events. If underlying assumptions prove inaccurate or unknown risks or uncertainties materialize, actual results could vary materially from Catalent's expectations and projections. Some of the factors that could cause actual results to differ include, but are not limited to, the following: the current or future effects of the COVID-19 pandemic or any global health developments on Catalent's and its customers' or suppliers' businesses; participation in a highly competitive market and increased competition that may adversely affect Catalent's business; demand for its offerings, which depends in part on its customers' research and development and the clinical and market success of their products; product and other liability risks that could adversely affect Catalent's results of operations, financial

condition, liquidity and cash flows; failure to comply with existing and future regulatory requirements; failure to provide quality offerings to customers could have an adverse effect on Catalent's business and subject it to regulatory actions and costly litigation; problems providing the highly exacting and complex services or support required; global economic, political and regulatory risks to Catalent's operations; inability to enhance existing or introduce new technology or service offerings in a timely manner; inadequate patents, copyrights, trademarks and other forms of intellectual property protections; fluctuations in the costs, availability, and suitability of the components of the products Catalent manufactures, including active pharmaceutical ingredients, excipients, purchased components and raw materials; changes in market access or healthcare reimbursement in the United States or internationally; fluctuations in the exchange rates of the U.S. dollar against other currencies; adverse tax legislative or regulatory initiatives or challenges or adjustments to Catalent's tax positions; loss of key personnel; risks generally associated with information systems; inability to complete any future acquisition or other transaction that may complement or expand its business or divest non-strategic businesses or assets and difficulties in successfully integrating acquired businesses and realizing anticipated benefits of such acquisitions; risks associated with timely and successfully completing, and correctly anticipating the future demand predicted for, capital expansion projects at existing facilities, offerings and customers' products that may infringe on the intellectual property rights of third parties; environmental, health and safety laws and regulations, which could increase costs and restrict operations; labor and employment laws and regulations or labor difficulties, which could increase costs or result in operational disruptions; additional cash contributions required to satisfy Catalent's existing pension plan obligations; substantial leverage that may limit its ability to raise additional capital to fund operations and react to changes in the economy or in the industry; and exposure to interest-rate risk to the extent of its variable-rate debt preventing it from meeting its obligations under its indebtedness. For a more detailed discussion of these and other factors, see the information under the caption "Risk Factors" in Catalent's Annual Report on Form 10-K for the fiscal year ended June 30, 2022, filed August 29, 2022. All forward-looking statements speak only as of the date of this release or as of the date they are made, and Catalent does not undertake to update any forward-looking statement as a result of new information or future events or developments except to the extent required by law.

SAREPTA FORWARD LOOKING STATEMENTS

This press release contains "forward-looking statements." Any statements contained in this press release that are not statements of historical fact may be deemed to be forward-looking statements. Words such as "believes," "anticipates," "plans," "expects," "will," "intends," "potential," "possible" and similar expressions are intended to identify

forward-looking statements. These forward-looking statements include statements regarding the parties' obligations and responsibilities under the agreement, meeting anticipated demand for SRP-9001, the potential approval of SRP-9001 and developing commercially scalable processes for additional gene therapy programs in Sarepta's pipeline.

These forward-looking statements involve risks and uncertainties, many of which are beyond Sarepta's control. Known risk factors include, among others: the expected benefits and opportunities related to the agreement may not be realized or may take longer to realize than expected; Sarepta may not be able to execute on its business plans and goals, including meeting its expected or planned regulatory milestones and timelines, clinical development plans, and bringing its product candidates to market, due to a variety of reasons, many of which may be outside of Sarepta's control, including possible limitations of company financial and other resources, manufacturing limitations that may not be anticipated or resolved for in a timely manner, regulatory, court or agency decisions, such as decisions by the United States Patent and Trademark Office with respect to patents that cover Sarepta's product candidates; the COVID-19 pandemic; and those risks identified under the heading "Risk Factors" in Sarepta's most recent Quarterly Report on Form 10-Q filed with the Securities and Exchange Commission (SEC) as well as other SEC filings made by Sarepta which you are encouraged to review.

Any of the foregoing risks could materially and adversely affect Sarepta's business, results of operations and the trading price of Sarepta's common stock. For a detailed description of risks and uncertainties Sarepta faces, you are encouraged to review the SEC filings made by Sarepta. We caution investors not to place considerable reliance on the forward-looking statements contained in this press release. Sarepta does not undertake any obligation to publicly update its forward-looking statements based on events or circumstances after the date hereof, except as required by law.

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Contact Us

RELATED TOPICS

Biologics Analytical Services

Exhibit I

sanofi

July 26, 2024

Mr. Douglas S. Ingram
President and Chief Executive Officer
Sarepta Therapeutics, Inc.
Sarepta Therapeutics Three, LLC
215 First St.
Cambridge, MA 02142

Re: Elevidys® Patent Infringement

Dear Mr. Ingram,

I write on behalf of Genzyme Corporation ("Genzyme") in relation to the manufacture and sale of Elevidys® (delandistrogene moxeparvovec-rokl) by Sarepta Therapeutics, Inc. and Sarepta Therapeutics Three, LLC's (collectively, "Sarepta").

Genzyme is at the forefront of converting the promise of gene therapy into innovative therapeutics. In particular, Genzyme has long engaged in pioneering research and development of recombinant adeno-associated virus ("rAAV") vector-based gene therapies, including their formulations and methods for their manufacture and characterization.

According to the Elevidys® label and Sarepta's public disclosures, Elevidys® is a non-replicating, rAAV vector of the serotype rh74 (AAVrh74) containing a micro-dystrophin transgene under the control of the MHCK7 promoter. Elevidys® "is an adeno-associated virus vector-based gene therapy indicated in individuals at least 4 years of age," approved "for the treatment of Duchenne muscular dystrophy (DMD) in patients who are ambulatory and have a confirmed mutation in the DMD gene" and "for the treatment of DMD in patients who are non-ambulatory and have a confirmed mutation in the DMD gene."

Having examined the available information regarding Elevidys®, we have concerns that Sarepta is infringing Genzyme's intellectual property by the manufacture and sale of Elevidys®. The formulation of Elevidys® and methods used in its manufacture, including those directed to the use of analytical ultracentrifugation ("AUC") techniques, disclosed in certain U.S. Food and Drug Administration materials, appear to infringe several Genzyme patents. By way of non-limiting example, we direct you to U.S. Patent Nos. 7,704,721, 9,051,542, 11,698,377, 12,013,326 and 12,031,894. We believe Sarepta currently lacks freedom to operate under Genzyme's patent estate.

Sarepta appears to take patent issues seriously and is undoubtedly aware of at least the '542 Patent, which has been the subject of prior litigation. As the '542 and '721 Patents expire next year, time is of the essence. Accordingly, we have filed a complaint in the District of Delaware, a copy of which is attached. We are willing to hold service for thirty (30) days to allow the parties to conduct license negotiations if Sarepta has an interest in early resolution of the dispute.

With respect to the AUC Patents, our understanding of current market reality is that the claimed process is necessary to make an approved rAAV gene therapy product. As those patents do not expire until 2036, and in an abundance of caution, we are willing to engage in dialogue on your position. If Sarepta is interested in such discussion, then we can discuss the contours. We call to your attention that the refusal to provide information relating to manufacturing in and of itself can form the basis to institute proceedings for patent infringement. *See K-Tech Telecomms., Inc. v. Time Warner Cable, Inc.*, 714 F.3d 1277, 1286 (Fed. Cir. 2013) ("A defendant cannot shield itself from a complaint . . . by operating in such secrecy that the filing of a complaint itself is impossible."). Indeed, the refusal to cooperate in providing manufacturing information can lead to the burden of proof on infringement shifting to the defendant under 35 U.S.C. § 295. *Syngenta Crop Protection, LLC v. Willowood, LLC*, No. 1:15-cv-274, 2017 WL 1133378, at *8 (M.D.N.C. Mar. 24, 2017), rev'd in part on other grounds, 944 F.3d 1344 (Fed. Cir. 2019).

Please advise whether Sarepta is interested in discussing a license. We are willing to license Genzyme's patents covering Elevidys® on fair and reasonable terms, but cannot of course, tolerate continued infringement of the Genzyme patent estate.

We look forward to your prompt reply.

Sincerely,

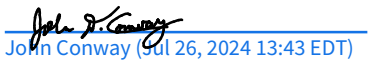
John Conway (Jul 26, 2024 13:43 EDT)

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Exhibit J

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October 31, 2024

VIA EMAIL

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Re: Elevidys® Patent Infringement

Dear Mr. Berdon and Mr. Livingstone,

I write to follow up on the July 26, 2024 letter from John Conway, on behalf of Genzyme Corporation ("Genzyme"), conveying Genzyme's concerns that Sarepta Therapeutics, Inc. and Sarepta Therapeutics Three, LLC (collectively, "Sarepta")'s manufacture and sale of Elevidys® (delandistrogene moxeparvovec-rokl) infringes Genzyme's intellectual property.

As stated in that letter, the Genzyme patent estate includes patents that claim analytical ultracentrifugation techniques covering a necessary process to make an approved rAAV gene therapy product like Elevidys®. Relevant here are U.S. Patent Nos. 12,013,326 and 12,031,894 (collectively, "AUC Patents"). As previously noted, the continued refusal to provide the pertinent information can form the basis for patent infringement proceedings. *See* July 26, 2024 J. Conway Ltr. to D. Ingram, p. 2 (citing cases).

Additionally, we have concerns that Sarepta has utilized Genzyme's patented methods for analyzing preparations of AAV particles and the viral proteins therein. Specifically, we call your attention to U.S. Patent Nos. 11,698,377 and 12,123,880 (with pending certificate of

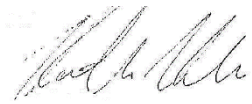
correction attached herein) (collectively, "AAV Analysis Patents"). Considering Sarepta's PCT Patent Publication No. WO 2021/138381 A1, for example, it appears Sarepta has used the processes described in the AAV Analysis Patents.

Genzyme's position is that Sarepta currently lacks freedom to operate, not only in view of U.S. Patent Nos. 9,051,542 and 7,704,721, currently asserted against Sarepta in C.A. No. 24-cv-882-RGA, but also the AUC Patents and the AAV Analysis Patents. If Genzyme is mistaken, please provide the details of Sarepta's non-infringement with respect to all past and current techniques for detection and analysis of AAV capsids in Elevidys®, including percent full, partial and empty capsids and the heterogeneity of the AAV capsids. Genzyme remains willing to engage in dialogue on Sarepta's position and to discuss the appropriate contours around any sharing of technical information.

Genzyme is deeply committed to protecting its intellectual property from continued infringement, but also appreciates the importance of its innovations and is similarly committed to facilitating access to them on fair and reasonable license terms. Please advise by November 8, 2024 whether Sarepta will provide the requested information and whether it is interested in further discussions about Genzyme's intellectual property rights or a license.

We look forward to your prompt reply.

Regards,



Katherine A. Helm, J.D., Ph.D.

UNITED STATES PATENT AND TRADEMARK OFFICE
P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/321,542	**10/23/2024 05:39:10 PM Z ET**	**15979-20141.10**

Title of Invention

METHODS FOR DETECTING AAV

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	12123880
CONFIRMATION #	9227	FILED BY	Aurelia Caparas
PATENT CENTER #	67694880	FILING DATE	05/22/2023
CUSTOMER #	89300	FIRST NAMED INVENTOR	Xiaoying JIN
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Seth Snyder

Documents

TOTAL DOCUMENTS: 2

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
Req_for_COC.pdf	2	Request for Certificate of Correction	90 KB
COC.pdf	2	Request for Certificate of Correction	159 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
Req_for_COC.pdf	B756F82D119506E726AC709729CC09DF2A817EAD1E6CD6231 759E8F1C556E38961026B239F8DC67DEA70B3CCE3C5AEB05 98678A84B6B638EA132254A2791EECB
COC.pdf	E5164879EE14458E68DAF0ACB1ABEFBA08E1717B4F7EA50A

004D759B0120CC0B08815AA4E0F51FD0E0FF27E577B51E998
F68CD8935F467B386BECFA14A39342D

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 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.: 15979-20141.10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Letters Patent of: Xiaoying JIN et al.

Patent No.: 12,123,880

Issued On: October 22, 2024

For: METHODS FOR DETECTING AAV

REQUEST FOR CERTIFICATE OF CORRECTION
PURSUANT TO 37 C.F.R. § 1.323

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Upon reviewing the above-identified patent, Patentee noted typographical errors which should be corrected. The certificate corrects claim 1 and claim 10 at clause b), which incorrectly refer to liquid chromatography/mass spectroscopy (LC/MS) rather than liquid chromatography/mass spectrometry (LC/MS). The error resulted from a preliminary amendment filed by Applicant on October 17, 2023, where the term liquid chromatography/mass spectroscopy was added rather than the term liquid chromatography/mass spectrometry in amended claims 3 and 130, which issued as claims 1 and 10, respectively. The issued patent includes other claims which correctly refer to mass spectrometry, including issued claims 8, 16, and 18. Moreover, the specification as filed consistently refers to liquid chromatography/mass spectrometry (LC/MS). *See*, for instance, the issued patent at col. 1, lines 30-31, col. 2, lines 37-38 and col. 2, line 49.

In the Claims:

At column 83, Claim 1, line number 14, please replace "spectroscopy" with -- spectrometry --; and

At column 83, Claim 10, line number 46, please replace "spectroscopy" with -- spectrometry --.

As this is an evident error, the correction does not involve new matter or require reexamination. Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

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. **15979-20141.10**.

Dated: October 23, 2024

Respectfully submitted,

Electronic signature: /Seth Snyder/
Seth Snyder
Registration No.: 60,243
MORRISON & FOERSTER LLP
425 Market Street
San Francisco, CA 94105-2482
+1 (415) 268-7046

PTO/SB/44 (09-07)
Approved for use through 03/31/2023. OMB 0651-0033
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.
(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 12,123,880

APPLICATION NO.: 18/321,542

ISSUE DATE : October 22, 2024

INVENTOR(S) : Xiaoying JIN

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

At column 83, Claim 1, line number 14, please replace “spectroscopy” with -- spectrometry --; and
At column 83, Claim 10, line number 46, please replace “spectroscopy” with -- spectrometry --.

MAILING ADDRESS OF SENDER (Please do not use Customer Number below):

MORRISON & FOERSTER LLP
425 Market Street
San Francisco, CA 94105-2482

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Exhibit K

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

GENZYME CORPORATION, Plaintiff, v. SAREPTA THERAPEUTICS, INC. and SAREPTA THERAPEUTICS THREE, LLC, Defendants.	)	C.A. No. 24-882 (RGA)

SAREPTA THERAPEUTICS, INC. AND SAREPTA THERAPEUTICS THREE, LLC'S OBJECTIONS AND RESPONSES TO PLAINTIFF GENZYME CORPORATION'S FIRST SET OF INTERROGATORIES (NOS. 1-10)

Pursuant to Rules 26 and 33 of the Federal Rules of Civil Procedure and the Local Rules of the United States District Court for the District of Delaware, Defendants Sarepta Therapeutics, Inc. and Sarepta Therapeutics Three, LLC (collectively, "Defendants" or "Sarepta") hereby respond and object to the First Set of Interrogatories to Defendants by Plaintiff Genzyme Corporation ("Plaintiff" or "Genzyme") as set forth below.

GENERAL OBJECTIONS

Sarepta's responses to Plaintiff's First Set of Interrogatories are made subject to and without waiver of any applicable objections. Sarepta incorporates each of the following General Objections into its responses to each of Plaintiff's interrogatories, whether or not expressly referred to in each response.

1. Sarepta objects to these interrogatories to the extent they seek to impose a duty upon Sarepta beyond those set forth in the Federal Rules of Civil Procedure or the Local Rules of the United States District Court for the District of Delaware.

2. Sarepta objects to these interrogatories to the extent they seek documents or information protected by the attorney-client privilege, attorney work product doctrine, or another privilege, immunity, or protection afforded by law.

3. Sarepta objects to these interrogatories to the extent that they are overly broad, unduly burdensome, oppressive, seek the production of information that is not relevant to any claim or defense, and are not proportional to the needs of the case.

4. Sarepta objects to these interrogatories to the extent they are vague or ambiguous.

5. Sarepta objects to these interrogatories to the extent they purport to assign meaning to words or terms different from their plain and ordinary meaning without expressly defining such words or terms.

6. Sarepta objects to these interrogatories to the extent they seek information already in Plaintiff's possession or to which Plaintiff has equal or greater access, information that is publicly available, and/or information that can be obtained through other means of discovery that are more convenient, less burdensome, or less expensive.

7. Sarepta objects to these interrogatories to the extent they are duplicative.

8. Sarepta objects to these interrogatories to the extent they seek information that is not in Sarepta's possession, custody, or control.

9. Sarepta objects to these interrogatories to the extent they seek information subject to confidentiality agreements, protective orders, or any other legal obligation pursuant to which Sarepta is required to protect or maintain the confidentiality of any third-party's documents or information. Inadvertent disclosure of any confidential information shall not operate as a waiver of any applicable confidentiality protections or obligations.

10. Sarepta objects to these interrogatories to the extent they seek patient-specific information that is subject to privacy laws and/or confidentiality agreements, including but not limited to, health information protected under state or federal privacy laws that Sarepta is required to maintain in confidence under the Health Insurance Portability and Accountability Act of 1996 ("HIPAA").

11. Sarepta objects to these interrogatories to the extent they could be construed to seek information protected by any foreign, federal, state, or local privacy laws, including, but not limited, to the extent they could be construed to seek employment records.

12. Sarepta objects to these interrogatories to the extent they seek information created after the date of filing the Complaint, purport to cover an unlimited time period, and/or are unbounded in time.

13. Sarepta objects to these interrogatories to they extent they seek to impose a duty beyond those set forth in the Federal Rules of Civil Procedure, the Local Rules of the United States District Court for the District of Delaware, the Default Standard for Discovery, Including Discovery of Electronically Stored Information, and 35 U.S.C. § 286.

14. Sarepta objects to these interrogatories to the extent they prematurely purport to limit Sarepta's contentions of law or fact before the deadlines set by the Scheduling Order (D.I. 31), the Local Rules, or any other rules or orders governing discovery in this litigation. Sarepta will comply with the Scheduling Order, the Local Rules, and any other rules, orders, or agreements of the parties with respect to discovery deadlines.

15. Sarepta reserves the right to supplement its responses, objections, or production if Sarepta identifies additional documents or information called for by these interrogatories.

OBJECTIONS TO DEFINITIONS AND INSTRUCTIONS

1. Sarepta objects to the definition of "Sarepta Therapeutics, Inc." to the extent it purports to include any entity other than Sarepta Therapeutics, Inc. Plaintiff's definition of "Sarepta Therapeutics, Inc." is overly broad, unduly burdensome, seeks the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, and seeks information that is not within Sarepta's possession, custody, or control, including to the extent it purports to include "affiliates, parents, divisions, joint ventures, licensees, franchisees, assigns, predecessors and successors in interest, and any of other legal entities, whether foreign or domestic, that are owned or controlled by Sarepta Therapeutics, Inc. or that own or control Sarepta Therapeutics, Inc., as well as all predecessors and successors in interest to such entities, and includes all officers, directors, current and former employees, counsel, agents, consultants, representatives, or any other persons acting on behalf of any of the foregoing."

2. Sarepta objects to the definition of "Sarepta Therapeutics Three, LLC" to the extent it purports to include any entity other than Sarepta Therapeutics Three, LLC. Plaintiff's definition of "Sarepta Therapeutics Three, LLC" is overly broad, unduly burdensome, seeks the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, and seeks information that is not within Sarepta's possession, custody, or control, including to the extent it purports to include "affiliates, parents, divisions, joint ventures, licensees, franchisees, assigns, predecessors and successors in interest, and any of other legal entities, whether foreign or domestic, that are owned or controlled by Sarepta Therapeutics Three, LLC or that own or control Sarepta Therapeutics Three, LLC, as well as all predecessors and successors in interest to such entities, and includes all officers, directors, current and former employees, counsel, agents, consultants, representatives, or any other persons acting on behalf of any of the foregoing."

3. Sarepta objects to the definitions of "Your," "Your," "Defendants," "Defendants'," "Sarepta," or "Sarepta's" as overly broad, unduly burdensome, and seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, including to the extent they purport to include any entities other than Sarepta Therapeutics, Inc. and Sarepta Therapeutics Three, LLC.

4. Sarepta objects to the definition of "Asserted Patent(s)" as vague and ambiguous to the extent it is not limited to the '542 and '721 patents and purports to include "any other patent Plaintiff asserts in this Litigation." To the extent Plaintiff's interrogatories are directed to any particular patent or application, Plaintiff should name that patent or application specifically in the interrogatory. To the extent that Plaintiff purports to include unspecified, additional patents within the scope of any interrogatory, Sarepta reserves all objections including, but not limited to, with respect to improper sub-parts.

5. Sarepta objects to the definition of "Asserted Claims(s)" as vague and ambiguous to the extent it is not limited to claims of the '542 and '721 patents and purports to include claims that "may be amended and supplemented from time to time as set forth in" Genzyme's Default Standard ¶ 4.c. disclosures that are due on April 16, 2025 per D.I. 31. To the extent that Plaintiff purports to include unspecified, additional claims within the scope of any interrogatory, Sarepta reserves all objections including, but not limited to, with respect to improper sub-parts.

6. Sarepta objects to the definitions of "Related Entity" or "Related Entities" as overly broad, unduly burdensome, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, and seeking information that is not within Sarepta's possession, custody, or control, including to the extent they purport to include "any parents, predecessors, subsidiaries, joint ventures, and other affiliated entities of

Sarepta Therapeutics, Inc. and/or Sarepta Therapeutics Three, LLC." Sarepta further objects to the definitions of "Related Entity" or "Related Entities" as vague and ambiguous with respect to "joint ventures, and other affiliated entities."

7. Sarepta objects to the definition of "Elevidys®" as overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous with respect to "all related and supplemental BLAs." Sarepta will interpret "Elevidys®" to mean the product that is the subject of BLA No. 125781.

8. Sarepta objects to the definition of "Elevidys® BLA" as overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous with respect to "all related and supplemental BLAs." Sarepta will interpret "Elevidys® BLA" to mean BLA No. 125781.

9. Sarepta objects to the definitions of "produce," "produced," "production," "manufacture," "manufactured," "manufacturing," "manufacturing process," and "manufacturing processes" overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous with respect to "the process of making" an Elevidys® product.

10. Sarepta objects to the definition of "identify" and "describe" with respect to a person to the extent that it seeks information protected by any foreign, federal, state, or local privacy laws. Sarepta further objects to the definitions of "identify" and "describe" as overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case to the extent it seeks information concerning dates and places of employment and general duties. Sarepta further objects to the definitions of "identify"

and "describe" "as used in connection with a person" as vague, ambiguous, and inconsistent with Plaintiff's definition of "Person."

11. Sarepta objects to the definitions of "identify" and "describe" with respect to a "company, corporation, association, partnership, joint venture, or other business or legal entity other than a natural person," as overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case to the extent it includes the "address of the principal place of business, state of incorporation, the location of any divisions, branches, or offices that are connected with or handled the matters referred to in the request or interrogatory, and the identity of the person acting or purporting to act on behalf of the business entity in connection with the matters referred to in the request or interrogatory."

12. Sarepta objects to the definitions of "identify" and "describe" as used in connection with an oral statement as overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case to the extent it seeks the "date and present location of said writing or mechanical or other recording" if the statement was memorialized.

13. Sarepta objects to the definitions of "identify" and "describe" as used in connection with "an act, event, or course of conduct" as overly broad, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous with respect to "complete description."

14. Sarepta objects to the definitions of "communication" as overly broad, unduly burdensome, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous, including to the extent it includes "every manner and means" of "statement, utterance, notation, disclaimer, transfer or

exchange of information of any nature whatsoever," "whether oral or written or whether face-to-face, by telephone, mail, personal delivery or otherwise, including, but not limited to, letters, correspondence, conversations, memoranda, dialogue, discussions, meetings, interviews, consultations, agreements and other understandings."

15. Sarepta objects to the definition of "concerning" and/or "relating to" as overly broad, unduly burdensome, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous. Sarepta will interpret "concerning" as "referring to, describing, evidencing, or constituting."

16. Sarepta objects to the definition of "considered" as overly broad, unduly burdensome, seeking the production of information that is not relevant to any claim or defense and not proportional to the needs of the case, vague, and ambiguous, including to the extent it includes "viewed, consulted, checked, referenced, confirmed, referred to."

17. Sarepta objects to Instruction No. 2 on the grounds that it seeks to impose a duty upon Sarepta beyond those set forth in the Federal Rules of Civil Procedure or the Local Rules of the United States District Court for the District of Delaware.

18. Sarepta objects to Instruction No. 3 to the extent it seeks to impose a duty upon Sarepta beyond those set forth in the Federal Rules of Civil Procedure, the Local Rules of the United States District Court for the District of Delaware, or the Default Standard for Discovery, Including Discovery of Electronically Stored Information.

19. Sarepta objects to Instruction No. 4 to the extent it seek to impose a duty upon Sarepta beyond those set forth in the Federal Rules of Civil Procedure, the Local Rules of the United States District Court for the District of Delaware, or the Default Standard for Discovery, Including Discovery of Electronically Stored Information. Documents withheld from production

on the basis of privilege will be logged in accordance with the Court's Scheduling Order and/or any subsequent order or agreement of the parties.

20. Sarepta objects to Instruction No. 5 to the extent it seeks to impose a duty upon Sarepta beyond those set forth in the Federal Rules of Civil Procedure, the Local Rules of the United States District Court for the District of Delaware, or the Default Standard for Discovery, Including Discovery of Electronically Stored Information.

OBJECTIONS AND RESPONSES TO INTERROGATORIES

INTERROGATORY NO. 1:

Describe in detail the complete legal and factual basis for any contention by You that Elevidys® and Your commercial manufacture and sale of Elevidys® has not infringed, is not infringing, and/or will not infringe any Asserted Claim of the Asserted Patents.

The detailed description should include, on a claim-by-claim basis, the identity of each claim limitation You contend is not met, either literally or under the doctrine of equivalents; the identity of all facts on which You base each such contention; the identity of each Person with knowledge of those facts and the knowledge You believe each such Person has; and the identity of each Document on which You base each such contention.

RESPONSE TO INTERROGATORY NO. 1:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome. Sarepta further objects to this Interrogatory as vague and ambiguous with respect to "complete legal and factual basis." Sarepta further objects to this Interrogatory as premature on the grounds that Plaintiff has not yet provided its Infringement Contentions, which are due on April 16, 2025 pursuant to the Scheduling Order (D.I. 31), and to the extent that it is dependent upon claim construction, the schedule for which is also set forth in the Scheduling Order. Sarepta further objects to this Interrogatory as premature to the extent that it seeks information that is properly the subject of expert discovery. Sarepta further objects to this

Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it calls for legal conclusions. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware. Sarepta further states that discovery recently commenced and is ongoing, and Sarepta will provide its non-infringement contentions according to the deadlines set under the Scheduling Order, and pursuant to the requirements under the Local Rules, and the Federal Rules of Civil Procedure. Sarepta reserves the right to supplement and/or amend this response, including, but not limited to, after Plaintiff discloses its Infringement Contentions, and/or after claim construction.

INTERROGATORY NO. 2:

Describe in detail the complete basis for Your contention that the Asserted Patents are invalid. The detailed description should include the identity of each statutory provision on which You rely and all facts on which You base the contention that the Asserted Patents are invalid under that provision; the identity of each person with knowledge of those facts and the knowledge You believe each such person has; and the identity of each Document on which You base each such contention.

For any contention that the Asserted Patents are anticipated or obvious over the Prior Art, the detailed description should also include the identity of each piece of Prior Art on which You rely; a claim-by-claim description of the basis for Your contention that the claim is anticipated or obvious in light of that Prior Art; and the identity of all facts on which You base the contention that each item of Prior Art on which You rely qualifies as Prior Art under 35 U.S.C. §§ 102 or 103.

The detailed description should also identify the level of education, training, specialty, and experience of the person having ordinary skill in the art for the subject matter described and claimed in the Asserted Patents.

RESPONSE TO INTERROGATORY NO. 2:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome. Sarepta further objects to this Interrogatory as vague and ambiguous with

respect to "the complete basis." Sarepta further objects to this Interrogatory as premature on the grounds that Sarepta's Initial Invalidity Contentions are not due until May 16, 2025 pursuant to the Scheduling Order (D.I. 31), and to the extent that it is dependent upon claim construction, the schedule for which is also set forth in the Scheduling Order. Sarepta further objects to this Interrogatory as premature to the extent that it seeks information that is properly the subject of expert discovery. Sarepta further objects to this Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it calls for legal conclusions. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware. Sarepta further states that discovery recently commenced and is ongoing, and Sarepta will provide its Initial Invalidity Contentions according to the deadlines set under the Scheduling Order, and pursuant to the requirements under the Local Rules, and the Federal Rules of Civil Procedure. Sarepta reserves the right to supplement and/or amend this response, including, but not limited to, after the due date for the Initial Invalidity Contentions and/or after claim construction.

Further answering, Sarepta states that it will comply with the Scheduling Order, which sets a May 16, 2025 due date for Sarepta's Initial Invalidity Contentions under Default Standard ¶ 4.d.

INTERROGATORY NO. 3:

For each month since the commercial launch of Elevidys® in the United States, identify Defendants' actual and forecasted sales through the life of each Asserted Patent by stating the number of prescriptions written for Elevidys® in the United States, the number of patients treated with Elevidys® in the United States, the number of units of Elevidys® sold (on a state-by-state basis), the number of patients treated with Elevidys® in the United States, the amount (in dollars) of sales of Elevidys® in the United States, and the amount of Sarepta's profits from sales of Elevidys® in the United States, and identify the amount of sales and profits attributable to sales after June 22, 2023.

RESPONSE TO INTERROGATORY NO. 3:

Sarepta objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case with respect to "the number of prescriptions written for Elevidys® in the United States," "the number of patients treated with Elevidys® in the United States," "the number of units of Elevidys® sold (on a state-by-state basis)," and "the number of patients treated with Elevidys® in the United States." Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case, and vague and ambiguous with respect to "commercial launch," "actual and forecasted sales," "life," "profits," and "profits attributable to sales." Sarepta further objects to this Interrogatory as seeking information that is not in Sarepta's possession, custody, or control. Sarepta further objects to this Interrogatory as seeking information subject to confidentiality agreements pursuant to which Sarepta is required to protect or maintain the confidentiality of a third-party's documents or information. Sarepta further objects to this Request to the extent that it seeks documents or information that is publicly available, and/or information that can be obtained through other means of discovery that are more convenient, less burdensome, or less expensive. Sarepta further objects to this Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states, pursuant to Rule 33(d) of the Federal Rules of Civil Procedure, that information

concerning Sarepta's revenue attributable to sales of Elevidys® made in the U.S. can be derived from documents that have been or will be produced in discovery, and the burden of deriving the answer will be substantially the same on Plaintiff as on Sarepta.

INTERROGATORY NO. 4:

Describe in detail all agreements entered into between and among any of the Defendants, any Related Entity, or any third party related to the manufacturing, sales, distribution, importation, or exportation of Elevidys®.

RESPONSE TO INTERROGATORY NO. 4:

Sarepta objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent it seeks information concerning agreements "related to the sales, distribution, importation, or exportation of Elevidys®." Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case, and vague and ambiguous with respect to agreements "related to" the manufacturing, sales, distribution, importation, or exportation of Elevidys®. Sarepta further objects to this Interrogatory as seeking information subject to confidentiality agreements pursuant to which Sarepta is required to protect or maintain the confidentiality of a third-party's documents or information. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states, pursuant to Rule 33(d) of the Federal Rules of Civil Procedure, that information concerning agreements about the manufacture of Elevidys® can be derived from documents that

have been or will be produced in discovery, and the burden of deriving the answer will be substantially the same on Plaintiff as on Sarepta.

INTERROGATORY NO. 5:

Identify the Persons most knowledgeable about any Communication with Federal Food and Drug Administration ("FDA") or any other regulatory agency related to Elevidys®, including but not limited to the preparation, filing, and prosecution of the Elevidys® BLA.

Describe in detail the involvement, role, or contribution of each such Person, and Identify all Documents, Things, and Communications related to each such Person's involvement, role, or contribution.

RESPONSE TO INTERROGATORY NO. 5:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as vague and ambiguous with respect to "most knowledgeable," and "related to." Sarepta also objects to this Interrogatory as vague and ambiguous with respect to "Federal" Food and Drug Administration. Sarepta further objects to this Interrogatory as seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent that it seeks information unrelated to the formulation of Sarepta's Elevidys® product. Sarepta further objects to this Interrogatory as seeking information that is not relevant to any parties' claims or defenses, not proportional to the needs of the case, vague and ambiguous to the extent that it seeks a detailed description of the "involvement, role, or contribution" of each person and the identification of "all" Documents, Things, and Communications "related to" each such Person's "involvement, role, or contribution." Sarepta further objects to this Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states that Meghan Brown, Vice President of Global Regulatory Strategy at Sarepta Therapeutics, is a person with knowledge of Sarepta's communications with FDA concerning the Chemistry, Manufacturing, and Controls (CMC) section of BLA No. 125781. Further answering, Sarepta states, pursuant to Rule 33(d) of the Federal Rules of Civil Procedure, that information responsive to this Interrogatory can be derived from documents that have been or will be produced in discovery, and the burden of deriving the answer will be substantially the same on Plaintiff as on Sarepta.

INTERROGATORY NO. 6:

Separately for each of the Asserted Patents, describe in detail the circumstances in which each Defendant first became aware of each of the Asserted Patents (or any patent application that later issued as one of the Asserted Patents), including, at minimum: how and when each Defendant first became aware of each of the Asserted Patents; the Person(s) so aware; any actions You took as a result of that awareness, including any freedom to operate analysis conducted; and any Documents or facts showing or reflecting each Defendant's belief of infringement or validity of each of the Asserted Patents from the time each Defendant first became aware of each of the Asserted Patents through trial in this action, including any opinions of counsel You obtained and/or the conclusions of any freedom to operate analysis conducted.

Your answer should identify by production number any Documents that concern, support, or refute Your answer.

RESPONSE TO INTERROGATORY NO. 6:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent that it seeks information concerning any "patent application that later issued as one of the Asserted Patents." Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case

to the extent that it asks Sarepta to describe "in detail" "the circumstances." Sarepta also objects to this Interrogatory as overly broad, unduly burdensome, not proportional to the needs of the case, and seeking to impose duties on Sarepta beyond those set forth in the Federal Rules of Civil Procedure or the Local Rules of the United States District Court for the District of Delaware, and the orders of this Court to the extent it seeks identification of "any" Documents or facts "showing or reflecting each Defendant's belief of infringement or validity of each of the Asserted Patents" "from the time each Defendant first became aware of each of the Asserted Patents through trial in this action." Sarepta also objects to this Interrogatory as overly broad, unduly burdensome, not proportional to the needs of the case, vague, ambiguous, and seeking to impose duties on Sarepta beyond those set forth in the Federal Rules of Civil Procedure or the Local Rules of the United States District Court for the District of Delaware, and the orders of this Court to the extent it seeks identification of "any" Documents that "concern, support, or refute" Sarepta's answer. Sarepta further objects to this Interrogatory as vague and ambiguous with respect to "circumstances," "first became aware," "showing or reflecting," "freedom to operate analysis," and "belief of infringement or validity." Sarepta further objects to this Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it calls for legal conclusions. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states, based on Sarepta's investigation to-date and facts currently known to it, that on or around August 22, 2023 Sarepta became aware of an August 18, 2023 Memorandum Opinion in District

of Delaware Civil Action No. 21-1736-RGA (D.I. 263) concerning the construction of certain claims terms in six patents, including U.S. Patent No. 9,051,542.

Sarepta further states, based on Sarepta's investigation to-date and facts currently known to it, that Sarepta became aware of U.S. Patent No. 7,704,721 on July 26, 2024, the filing date of the initial complaint in this case.

INTERROGATORY NO. 7:

Identify, including by Bates number, all patent license agreements that You contend provide terms comparable to those upon which You would have licensed the Asserted Patents.

RESPONSE TO INTERROGATORY NO. 7:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as vague and ambiguous with respect to "terms comparable." Sarepta further objects to this Interrogatory as seeking information subject to confidentiality agreements pursuant to which Sarepta is required to protect or maintain the confidentiality of a third-party's documents or information. Sarepta further objects to this Interrogatory as premature to the extent that it seeks information that is properly the subject of expert discovery. Sarepta further objects to this Interrogatory to the extent that it calls for legal conclusions. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

INTERROGATORY NO. 8:

Identify the Persons most knowledgeable about any manufacture, research, development, testing, and analysis of Elevidys® and/or any other delandistrogene moxeparvovec-rokl formulation considered, evaluated, or tested by Defendants during the course of the development of Elevidys®, including a detailed description of the involvement, duration of involvement, role, or contribution of each such Person, and an identification of all Documents, Things, and Communications related thereto.

RESPONSE TO INTERROGATORY NO. 8:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent that it seeks information unrelated to the formulation of Sarepta's Elevidys® product. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent that it seeks information concerning "any" "manufacture, research, development, testing, and analysis" of Elevidys® and/or "any other delandistrogene moxeparvovec-rokl formulation." Sarepta further objects to this Request as vague and ambiguous with respect to "most knowledgeable," "testing, and analysis," and "considered, evaluated, or tested." Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case, vague, and ambiguous with respect to "detailed" description of "involvement," "role," and "contribution." Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case, vague, and ambiguous to the extent it seeks identification of "all" Documents, Things, and Communications "related" thereto. Sarepta further objects to this Interrogatory as seeking information subject to confidentiality agreements pursuant to which Sarepta is required to protect or maintain the confidentiality of a third-party's documents or information. Sarepta further objects to this Interrogatory as containing multiple discrete sub-

parts. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states, pursuant to Rule 33(d) of the Federal Rules of Civil Procedure, that information concerning manufacture, research, development, testing, and analysis of the formulation of Sarepta's Elevidys® product can be derived from documents that have been or will be produced in discovery, and the burden of deriving the answer will be substantially the same on Plaintiff as on Sarepta.

INTERROGATORY NO. 9:

Describe in detail the bases for Your contentions regarding the amount of damages owed to Genzyme, including (a) the damages amount for past infringement and future infringement; (b) the methodology of calculating damages (e.g., reasonably royalty or some other measure); (c) the nature and amount of the royalty base (including all products and acts that comprise the royalty base); (d) the date(s) of the hypothetical negotiation that should be used for evaluating a reasonable royalty; (e) the nature of and basis for any alleged apportionment methodology for calculating damages; (f) the royalty rate for calculating damages in the form of a reasonable royalty, including Your position on each of the factors set forth in *Georgia-Pacific Corp. v. U.S. Plywood Corp.*, 318 F. Supp 1116, 1120 (S.D.N.Y. 1970); (g) any license agreements, cross-licenses, settlement agreements, covenants-not-to-sue, other agreements, license proposals, offers to license, or negotiations that You contend may be relevant to the calculation of damages; and identify the Persons most knowledgeable about the facts supporting Your contentions.

RESPONSE TO INTERROGATORY NO. 9:

Sarepta objects to this Interrogatory to the extent that it seeks information that is protected from discovery by the attorney client privilege, the work product doctrine and/or any other privilege or protection. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case, case, including to the extent that it identification of "all products and acts." Sarepta further objects to this Interrogatory as vague

and ambiguous with respect to "bases," "nature," "products and acts," "most knowledgeable," and "facts supporting." Sarepta further objects to this Interrogatory as seeking information that is not in Sarepta's possession, custody, or control. Sarepta further objects to this Interrogatory as seeking information subject to confidentiality agreements pursuant to which Sarepta is required to protect or maintain the confidentiality of a third-party's documents or information. Sarepta further objects to this Interrogatory as premature to the extent that it seeks information that is properly the subject of expert discovery. Sarepta further objects to this Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it calls for legal conclusions. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states that no damages are owed to Genzyme. Further responding, if the Asserted Patents are found to be valid and infringed, any damages should be no more than necessary to compensate Genzyme for any infringement. As an initial matter, Plaintiff bears the burden of proving damages. *See Lucent Techs., Inc. v. Gateway, Inc.*, 580 F.3d 1301, 1324 (Fed. Cir. 2009). Any award of damages for patent infringement is compensatory: "[u]pon finding for the claimant the court shall award the claimant damages adequate to compensate for the infringement, but in no event less than a reasonable royalty for the use made of the invention by the infringer, together with interest and costs as fixed by the court." 35 U.S.C. § 284. "[T]he purpose of compensatory damages is not to punish the infringer, but to make the patentee whole." *See Pall Corp. v. Micron Separations, Inc.,* 66 F.3d 1211, 1223 (Fed. Cir. 1995). Further, it is improper to base damages on the entire value of a product if the patented feature is merely one of many elements

of the product. *See, e.g.*, *LaserDynamics, Inc. v. Quanta Computer, Inc*., 694 F.3d 51, 68 (Fed. Cir. 2012); *Lucent Techs.,* 580 F.3d at 1332. It is also improper for a patentee to charge royalties for use of the patented invention after the patent term has expired. *See Brulotte v. Thus Co.,* 379 U.S. 29 (1964); *see also Kimble v. Marvel Entertainment, LLC.*, 576 U.S. 446 (2016).

Sarepta reserves the right to supplement and/or amend this response, including, but not limited to, after such time as Plaintiff discloses its damages contentions.

INTERROGATORY NO. 10:

Identify and describe in detail how and to whom You advertise, promote, or market Elevidys®, or functions or features thereof, including the identity and role of the Persons most knowledgeable about Your advertising, promotion, and marketing of Elevidys®.

RESPONSE TO INTERROGATORY NO. 10:

Sarepta objects to this Interrogatory as seeking information that is not relevant to any claim or defense and not proportional to the needs of the case. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent that it seeks description "in detail' of "how" and "to whom" Sarepta advertises, promotes, or markets Elevidys®. Sarepta further objects to this Interrogatory as overly broad and unduly burdensome, and seeking information that is not relevant to any parties' claims or defenses and not proportional to the needs of the case to the extent that it seeks information unrelated to the formulation of Sarepta's Elevidys® product. Sarepta further objects to this Interrogatory as vague and ambiguous with respect to describe "in detail," "advertise, promote, or market," "functions or features," "role," and "most knowledgeable." Sarepta further objects to this Interrogatory as containing multiple discrete sub-parts. Sarepta further objects to this Interrogatory to the extent that it seeks to impose burdens on Sarepta beyond those required by

the Federal Rules of Civil Procedure and/or the Local Rules of the United States District Court for the District of Delaware.

Subject to and without waiver of the foregoing general and specific objections, Sarepta states that Kerry Siracusa, Senior Director of Marketing at Sarepta Therapeutics, Inc. is a person with knowledge of ELEVIDYS® marketing. Further answering, Sarepta states, pursuant to Rule 33(d) of the Federal Rules of Civil Procedure, that information concerning ELEVIDYS® marketing can be derived from documents that have been or will be produced in discovery, and the burden of deriving the answer will be substantially the same on Plaintiff as on Sarepta.

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April 4, 2025

CERTIFICATE OF SERVICE

I hereby certify that on April 4, 2025, copies of the foregoing were caused to be served upon the following in the manner indicated:

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Exhibit L

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Identification of Factors that Contribute to Recombinant AAV2 Particle Aggregation and Methods to Prevent Its Occurrence during Vector Purification and Formulation

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Aggregation of recombinant AAV2 results in reduced yield during purification and may have deleterious effects on vector transduction efficiency, biodistribution and immunogenicity following *in vivo* administration. Studies to elucidate the mechanism of vector aggregation and methods to prevent its occurrence are reported. In excipient screening studies, the sugars sorbitol, sucrose, mannitol, trehalose, or glycerol at concentrations of up to 5% (w/v), or surfactants Tween 80 or Pluronic F68, did not prevent aggregation. Aggregation was prevented by the use of various salts at concentrations corresponding to solution ionic strengths of >200 mM. AAV2 vectors purified by double cesium chloride gradient centrifugation, cation-exchange chromatography, or combined chromatography and gradient centrifugation each demonstrated a similar requirement for ionic strength to prevent aggregation. AAV2 vectors concentrated to 6.7×10^{13} vector genome (vg)/mL in neutral-buffered isotonic saline resulted in 59 ± 6.0% recovery of nonaggregated material compared to 96 ± 4.4% recovery in an isotonic formulation with elevated ionic strength. The latter showed no aggregation following storage or after 10 freeze–thaw cycles at −20°C. AAV2 vectors stored for an extended period in an elevated ionic strength formulation retained a high infectivity titer (13 vg/infectious unit) and transduction efficiency. Nuclease digestion of purified AAV2 vectors reduced aggregation, implicating trace amounts of vector surface nucleic acids in interparticle binding.

Key Words: adeno-associated virus, vector aggregation, ionic strength

Introduction

Recombinant adeno-associated virus (rAAV) is a promising vector for human gene transfer [1–3]. A member of the *Dependovirus* genus of the parvoviruses, AAV Type 2 (AAV2) is composed of a single-strand DNA molecule of 4680 nucleotides encoding replication (*rep*) and encapsidation (*cap*) genes flanked by inverted terminal repeat sequences [4]. The genome is packaged by three capsid proteins (VP1, VP2, and VP3), which are amino-terminal variants of the *cap* gene product. The icosahedral virus particle has a diameter of ~26 nm. A high-resolution crystal structure of AAV2 has been reported [5].

The solubility of purified AAV2 particles is limited, and aggregation of concentrated AAV2 vectors has been reported [6–9]. Aggregation can lead to purification losses and inconsistencies in the testing of purified vector. The *in vivo* administration of AAV2 vectors to certain sites, such as the central nervous system, may require small volumes of concentrated vector, and the maximum achievable dose is limited due to low vector solubility. Vector aggregation is also likely to influence biodistribution following *in vivo* administration and may cause unwanted immune responses to vectors, as has been reported for proteins [10]. Reports of immune responses that limit transgene expression following AAV vector administration in preclinical [11–13] and clinical [14] studies emphasize the need to address factors that may contribute to vector immunogenicity. Hence, an important objective for the development of AAV2 vectors is to optimize vector purification methods and formulations to prevent aggregation when concentrated vector stocks are prepared. To achieve this objective, the biochemical

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mechanisms that contribute to aggregation should be elucidated. In this study we have investigated AAV2 vector aggregation by examining the influence of different classes of excipients and by identifying impurities that contribute to aggregation. Methods to prevent the aggregation of AAV2 vectors are described.

Results

Excipient Screening by Dynamic Light Scattering

Initial screening experiments were performed to identify classes of excipients that could reduce aggregation and thereby provide information on the mechanism of AAV vector aggregation. We observed that vector aggregation could be caused by the dilution of our purified AAV2 vector preparations with a low-concentration buffer (10 mM sodium phosphate, pH 7.2). Based on this "dilution-stress" method, we screened for excipients that, when included in the diluent, were able to prevent vector aggregation. For this screening, aggregation was measured by dynamic light scattering (DLS), a method that is highly sensitive, requires only small volumes (20 μL) of sample, and provides a semiquantitative measure of aggregation adequate for comparison studies. Excipients examined included selected inorganic salts, amino acids, uncharged simple carbohydrates, and surfactants. The results are shown in Table 1. Charged excipients (inorganic salts and amino acids) were able to prevent aggregation when present at sufficient concentrations. However, concentrations required to prevent vector aggregation varied, ranging from 180 mOsm for magnesium sulfate to 320 mOsm for sodium chloride. The amino acids Arg, Asp, Glu, Gly, His, and Lys were each found to be unable to prevent aggregation when initially screened at 200 mOsm. Three amino acids (Lys, Asp, and Glu) were examined at higher concentrations and found to prevent aggregation at 300–320 mOsm. Several carbohydrates were tested at concentrations of up to 5% w/v and found to have no effect on vector particle aggregation. For example, 5% w/v glycerol (543 mOsm) did not prevent aggregation under the dilution-stress method. The surfactants Pluronic F68 (to 10% w/v) and Polysorbate 80 (to 1% w/v) similarly had no effect.

TABLE 1: Screening for excipients that prevent AAV2 vector aggregation using a dilution-stress method

Excipient	Osm required to prevent aggregation (max tested)
Magnesium sulfate	180 mOsm
Sodium citrate	220 mOsm
Sodium chloride	320 mOsm
Sodium phosphate	220 mOsm
Sodium sulfate	220 mOsm
Arginine	NIA (200 mOsm)
Aspartic acid	320 mOsm
Glutamic acid	320 mOsm
Glycine	NIA (200 mOsm)
Histidine	NIA (200 mOsm)
Lysine	300 mOsm
Glycerol	NIA (5% w/v, 543 mOsm)
Iodixanol	NIA (5% w/v, 32 mOsm)
Mannitol	NIA (5% w/v, 275 mOsm)
Sorbitol	NIA (5% w/v, 275 mOsm)
Sucrose	NIA (5% w/v, 146 mOsm)
Trehalose	NIA (5% w/v, 146 mOsm)
Pluronic F68	NIA (10% w/v, 12 mOsm)
Polysorbate 80	NIA (1% w/v)

NIA, no inhibition of aggregation.

Vector Aggregation at Reduced Ionic Strength

A more detailed analysis of AAV2 vector aggregation as a function of the concentration of selected excipients was performed. Shown in Fig. 1A is the dependence of aggregation on the osmolarity of these excipients. For charged excipients a concentration-dependent inhibition of aggregation was observed. Salts of multivalent ions were required at lower concentrations to prevent aggregation than was NaCl. For example, magnesium sulfate at ~200 mOsm prevented aggregation, while NaCl was required at ~350 mOsm to achieve a similar effect. Sodium salts of citrate, sulfate, and phosphate were intermediate in their potency. These data suggested that the ionic strength (μ) of the solution, a parameter that depends on charge valency as well as concentration, was the excipient characteristic affecting vector aggregation. In Fig. 1B, the data were plotted to show vector aggregation as a function of the calculated ionic strength of solution for each excipient. This transformation showed that the dependence of vector aggregation on ionic strength was the same regardless of which salt was used, and aggregation was prevented in all cases in which the ionic strength was ~200 mM or greater.

Effect of Purification Method on AAV Vector Aggregation

Recombinant AAV2 purified using different methods (e.g., density-gradient purification versus ion-exchange chromatography) would be expected to have differing impurity profiles. To investigate the effect of purification method, aggregation as a function of ionic strength was measured for vectors purified by three methods. In these studies, NaCl was used to vary ionic strength. As shown in Fig. 1C, AAV2 vectors purified by double cesium chloride gradient ultracentrifugation (Method 1), by cation-exchange column chromatography (Method 2), or by combined column and CsCl gradient ultracentrifugation (Method 3) each aggregated to a similar degree at low ionic strengths. In contrast, AAV2-FIX purified by the column method and then subjected to an additional nuclease digestion step (Method 2 plus nuclease) to further degrade and remove DNA impurities showed a reduced degree of aggregation at low ionic strengths.

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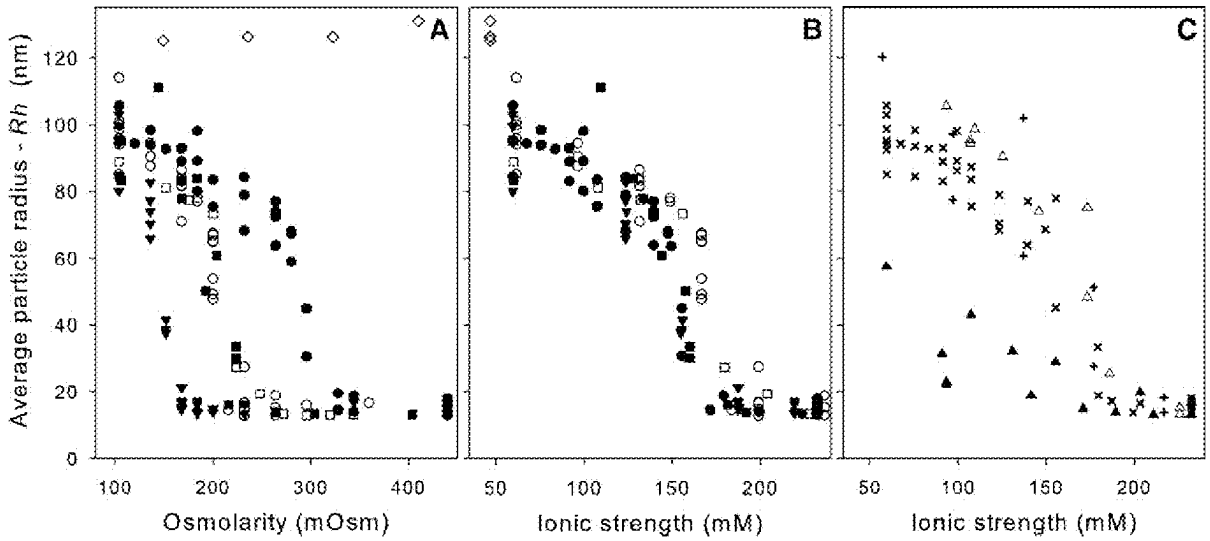

FIG. 1. Dependence of AAV2 vector aggregation on osmolarity and ionic strength of selected excipients and on the method of purification. The average particle radius of AAV2-FIX vectors was measured by DLS following vector dilution in varying concentrations of excipients buffered with 10 mM sodium phosphate, pH 7.5. (A) Aggregation of vectors purified by Method 3 (see Materials and Methods) as a function of the osmolarity of sodium chloride (●), sodium citrate (○), sodium phosphate (■), sodium sulfate (□), magnesium sulfate (▼), and glycerol (◇). (B) Vector aggregation as a function of the ionic strengths of the same solutions. (C) AAV2 vector aggregation as a function of ionic strength of sodium chloride for vectors purified by Method 1 (double CsCl gradient) (+); Method 2 (cation exchange chromatography) (△); Method 2 plus nuclease digestion (▲); or Method 3 (chromatography plus CsCl gradient) (×).

Nuclease digestion performed at an earlier stage of the purification process (clarified HEK cell lysate as in Method 1) did not reduce the aggregation of subsequently purified vector even though the amount of residual nonvector DNA was not reduced significantly following additional nuclease digestion of the purified vector when measured by real-time quantitative (Q)-PCR.

Effects of Ionic Strength and Nuclease on a Preparative Scale

The preceding results describing the dependence of vector aggregation on ionic strength and nuclease treatment were performed on an analytical scale, employing a method to measure aggregation (DLS) that is semiquantitative. The effects of elevated ionic strength and nuclease treatment on AAV2 vector aggregation were next tested on a larger scale using methods to induce and quantify vector aggregation relevant to preparative-scale vector purification. Purified AAV vectors were diafiltered into solutions of varying ionic strengths, the volume was reduced to reach high target vector concentrations, and aggregation was then assessed by our measuring vector recovery following filtration of the product using a 0.2-μm filter. Aliquots from a single pool [1.7×10^{15} vector genome (vg) in 91 mL ~3 M CsCl, 1.9×10^{13} vg/mL] of AAV2-AADC vector purified by Method 1 through the second CsCl gradient centrifugation step were used as the starting material in the experiments described in Table 2. Tangential-flow filtration using hollow fibers was used for diafiltration because this method is scalable and enabled the accurate preparation of small volumes (minimum ~1.4 mL) of concentrated vectors. In Experiment 1, three hollow-fiber units were used to diafilter AAV2-AADC vector into formulations CF, TF1, or TF2 (Control Formulation, Test Formulation 1, Test Formulation 2), respectively, and then the volume was reduced to a value corresponding to 2.5×10^{13} vg/mL. Formulation ionic strengths and vector recoveries following 0.2-μm

TABLE 2: AAV vector recovery on a process scale following diafiltration and concentration in control and elevated-ionic-strength formulations, followed by 0.2-μm filtration

Experiment	Formulation	μ (mM)	Target (vg/mL)	Actual (vg/mL)	Recovery % (RSD)
1	CF	160	2.5×10^{13}	1.93×10^{13}	77 (6.6)
	TF1	310	2.5×10^{13}	2.38×10^{13}	95 (7.4)
	TF2	510	2.5×10^{13}	2.33×10^{13}	93 (7.4)
2	CF	160	6.7×10^{13}	3.98×10^{13}	59 (6.0)
	TF2	510	6.7×10^{13}	6.42×10^{13}	96 (4.4)
3	CF (−Bz)	160	3.6×10^{13}	2.46×10^{13}	68 (11)
	CF (+Bz)	160	3.6×10^{13}	3.29×10^{13}	91 (12)

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filtration are shown in Table 2. Recoveries using both elevated ionic strength formulations TF1 (95 ± 7.4%) and TF2 (93 ± 7.4%) were significantly higher than that using CF (77 ± 6.6%). In Experiment 2, AAV2-AADC was concentrated to a higher target value (6.7×10^{13} vg/mL) in CF or TF2. Vector recovery using TF2 (96 ± 4.4%) was again significantly higher than recovery using CF (59 ± 6.0%). Within the variability of the assays used, vector was recovered fully at both target concentrations (Experiments 1 and 2) using TF2, indicating that aggregation was prevented. In contrast, significant aggregation was observed at these target concentrations using CF, and the extent of aggregation (i.e., loss following 0.2-μm filtration) was proportional to the target vector concentration. Formulation TF1 was not used in Experiment 2 because of its poor stability following freeze–thaw cycling (Table 3), coupled with the limited supply of vector. In Experiment 3, the effect of prior nuclease digestion of purified vector on aggregation was examined. In the absence of nuclease digestion, recovery of AAV2-AADC was 68 ± 11%, indicating a degree of aggregation consistent with that observed using CF in Experiments 1 and 2. In contrast, purified vector treated with nuclease and then concentrated in CF gave a greater recovery (91 ± 12%). The results obtained on a preparative scale are concordant with the effect of nuclease on vector aggregation observed using the analytical-scale dilution-stress method (Fig. 2), confirming that efficient nuclease digestion of purified AAV2 vectors results in reduced aggregation.

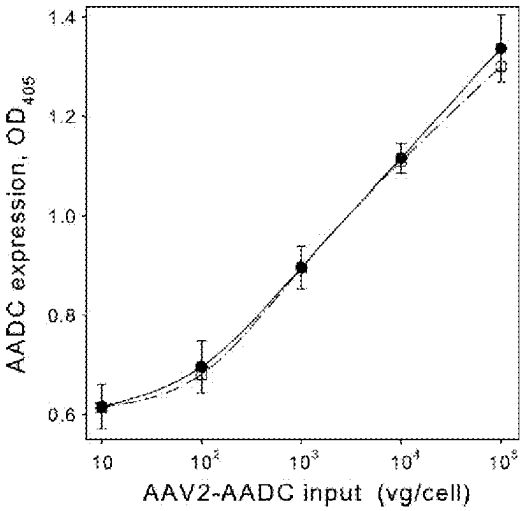

FIG. 2. Transduction by AAV2 vectors prepared and stored in elevated-ionic-strength formulation TF2. D7/4 cells were infected with AAV2-AADC prepared in control (●) or TF2 formulations (□) at 10-fold serial multiplicities. After 72 h, the concentration of AADC in each well was measured by ELISA. Error bars indicate the standard deviation of the average value ($n = 5$) measured at each dilution.

Stability of AAV2 Vectors Following Storage Or Freeze–Thaw Cycling

The concentrated vectors prepared in CF, TF1, and TF2 (Table 2, Experiment 1) were subjected to a short stability study to investigate whether aggregation would occur during refrigerated storage or following multiple freeze–thaw (F/T) cycles. Aggregation was assessed by DLS, and *Rh* values >20 nm were deemed to indicate the occurrence of aggregation. As shown in Table 3, AAV2-AADC vector prepared in CF showed some aggregation after 5 days of storage at 4°C, as well as following one or more F/T cycles at −20 or −80°C. For vector prepared in TF1, no aggregation occurred after 5 days at 4°C; however, aggregation occurred after one or more F/T cycles at −20 or −80°C, as indicated by the high DLS signal intensity (too high to measure in neat samples). Visual inspection indicated slight cloudiness in these samples, which is consistent with aggregation. For vector prepared in TF2, no aggregation was observed after storage at 4°C or follow-up to 10 F/T cycles at −20°C. Some aggregation was observed after 5 and 10 F/T cycles at −80°C.

Functional Studies

As described above, the high-ionic-strength isotonic formulation TF2 effectively prevented vector aggregation during concentration and storage and represented a promising candidate for further study. An important question was whether preparation and storage of the vector in high-ionic-strength TF2 would adversely affect its functional activity. To assess this, assays were performed to measure the infectious titer and transduction

TABLE 3: Stability of AAV2 vectors prepared in control and elevated-ionic-strength formulations after 4°C storage or freeze–thaw (F/T) cycling

	Particle radius—*Rh* (nm)							
		4°C	−20°C			−80°C		
Formulation	Pre[a]	5 days	1 F/T	5 F/T	10 F/T	1 F/T	5 F/T	10 F/T
CF[b]	14.5	27.0	22.4	56.1	94.5	20.6	57.5	141
TF1	13.8	16.3	TH[c]	TH	TH	TH	TH	TH
TF2	13.8	14.4	14.2	14.0	14.1	13.8	21.3	50.9

[a] Pre, DLS radius measured immediately following 0.2-μm filtration.
[b] Vector concentrations (vg/mL): CF, 1.93×10^{13}; TF1, 2.38×10^{13}; TF2, 2.33×10^{13}.
[c] TH, signal intensity too high to measure.

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efficiency of vectors prepared and stored for an extended period of time in TF2. For infectivity, a highly sensitive assay capable of detecting single infectious events was used [15]. AAV2-AADC prepared in TF2 at a concentration of 6.4×10^{13} and stored at 4°C for 45 days was assayed and determined to have a vector genome-to-infectious unit ratio (vg/IU) of 13 compared to a value of 16 vg/IU for the reference vector. This difference is not significant given the reported variability of the assay (RSD ~50%). Transduction efficiency was assessed by measuring the expression of AADC protein by ELISA following the transduction of D7/4 cells. As shown in Fig. 2, at vector inputs ranging from 10 to 10^5 vg/cell there was no significant difference between vector prepared in TF2 and the reference control. Together, these data indicate that preparation and storage of AAV2 vectors in high-ionic-strength TF2 does not have a detrimental effect on vector infectivity or transduction efficiency.

Discussion

Critical to the success of clinical gene transfer vectors is the development of purification methods and final product formulations that ensure high safety, consistency, and potency appropriate for preclinical and clinical applications. An important requirement is to maintain product solubility during purification and storage, as is well established for protein therapeutics [16–19]. For AAV2, vector particle aggregation is a problem that has been previously recognized [6–9]. Factors influencing vector aggregation, including the intrinsic characteristics of the virus particle as well as the role of trace impurities, need to be well defined to optimize vector purification methods and product formulation and storage conditions.

In the current study we found that some degree of aggregation of AAV2 vectors purified by our laboratory occurred in neutral-buffered solutions such as phosphate- and Tris-buffered saline when particle concentrations reached approximately $1–2 \times 10^{13}$ particles/mL. In studies that aimed to increase vector solubility in low-ionic-strength solutions to support crystal structure determination, Xie and colleagues reported that 25% (w/v) glycerol enabled concentration of AAV2 to very high concentrations ($4.4–18 \times 10^{14}$ particles/mL) [9]. In our preliminary screen of excipients, we tested glycerol and other sugars at concentrations of up to 5% and did not observe a reduction of vector aggregation induced by low ionic strength. The mechanism by which glycerol improves the solubility of AAV2 may be most effective at higher concentrations. Croyle and colleagues reported a significant loss of the titer of AAV and adenovirus, possibly due to aggregation, following multiple freeze–thaw cycles in sodium phosphate buffer [6]. The results of our freeze–thaw stability study using sodium phosphate are consistent with their findings. We found that while 150 mM sodium phosphate provided sufficient ionic strength to prevent aggregation during preparation and nonfrozen storage of concentrated AAV2-AADC vector, even a single freeze–thaw cycle resulted in aggregation. Particle aggregation is also an unresolved issue for adenovirus. A field-use stability study of a recently established adenovirus reference material was reported [20] in which variable levels of virus aggregation were reported.

We have shown that solution ionic strength is a parameter affecting the solubility of our AAV2 vector preparations, implicating ionic interactions between virus particles in aggregation. The observation that elevated ionic strength increased AAV2 vector solubility regardless of the identity of the charged excipient supports the hypothesis that ionic strength per se, rather than interactions involving a specific ion species, is important. The low solubility of AAV2 particles might be caused by its highly symmetrical structure in conjunction with the stabilizing effect of interactions between oppositely charged moieties such as amino acid side chains on neighboring particles. A pH dependence of AAV2 vector aggregation that is consistent with the participation of charged-vector-surface amino acids has been reported previously [9,21]. However, in the current study we observed that the addition of free amino acids with charged side chains prevented AAV2 vector aggregation only at ionic strengths at which aggregation was prevented using other salts. The absence of a specific effect using soluble amino acids suggests that other mechanisms contributed to interparticle interactions in our studies. Vector aggregation at low ionic strength was found to be reduced by nuclease treatment of already purified vector particles, suggesting that nucleic acid impurities (e.g., host cell and plasmid DNA fragments) associated with the surface of virus particles can form ionic bonds to neighboring particles. We previously reported [22] that AAV2 vector stocks prepared by transient transfection and purified by density gradient ultracentrifugation contained approximately 46 pg/10^9 vg of nonvector DNA (plasmid and mammalian). This nucleic acid was found to be resistant to nuclease digestion, and we concluded that it was packaged. However, the observation reported here that nuclease treatment of our purified AAV2 vectors reduced aggregation implicates residual vector surface nucleic acids. An explanation for this discrepancy is that while most of the impurity DNA in our preparations is packaged, a small amount of DNA that cannot be resolved by our Q-PCR assay is present on the vector surface and contributes to aggregation. For a preparation with 46 pg nonvector DNA per 10^9 vg, we estimate that the amount of surface-associated nucleic acid is less than the standard deviation of the Q-PCR assay (RSD ~10%), therefore <4.6 pg/10^9 vg.

The conditions that we used for Benzonase digestion of crude cell lysates did not achieve the degree of removal

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of vector surface DNA required to prevent aggregation in subsequently purified vector. This may pertain specifically to our protocol, which required an elevated salt concentration to efficiently extract vector from cells, conditions not optimal for Benzonase activity. Nuclease treatment of purified vector was effective in reducing aggregation and may provide a useful purification step for optimizing the stability of concentrated vectors preparations. This use of a nuclease would require additional steps to ensure its subsequent removal, such as diafiltration with an appropriate membrane that retains vector but not the nuclease molecule.

Commonly used buffered-saline solutions have ionic strengths (μ ~150 mM) that may be insufficient to prevent aggregation of concentrated AAV2 with trace amounts of vector surface-associated nucleic acids. In our screening studies, an ionic strength of ≥200 mM was required to prevent aggregation at the vector particle concentrations examined, and higher ionic strengths (300–500 mM) may be preferred for optimal solubility and stability. Formulations for preclinical and clinical studies should be approximately isotonic, especially for *in vivo* administration of vector to sites at which solute diffusion may be slow. The exponential relationship of ionic strength with charge valency can be used to achieve this objective. Compared to monovalent salts such as sodium chloride, salts with multiple valencies (e.g., some salts of sulfate, citrate, and phosphate) that are established parenteral excipients (www.accessdata.fda.gov/cder/iig/index.cfm) used at isotonic concentrations can provide higher ionic strengths and thereby enhance the solubility of concentrated AAV2 vectors. For example, the sodium citrate formulation (TF2) characterized in these studies was isotonic (315 mOsm) and provided an ionic strength (510 mM) that enabled AAV2 vector concentration to 6.4×10^{13} vg/mL without evidence of aggregation.

In summary, our studies show that trace amounts of nucleic acid impurities associated with the vector surface can contribute to ionic interactions between vector particles contributing to AAV2 vector aggregation. Efficient removal of residual vector surface nucleic acids during purification and the use of elevated-ionic-strength solutions during AAV2 vector purification and formulation are useful strategies to achieve stable, concentrated solutions of AAV2 vectors.

Materials and Methods

AAV purification. AAV2 vectors expressing human coagulation factor IX (FIX) or human amino acid decarboxylase (AADC) were produced by triple transfection of HEK293 cells as previously described [23], with modifications. For large-scale preparations, cells were cultured and transfected in 850-cm^2 roller bottles (Corning). Vectors were purified by one of three methods. In purification Method 1, transfected HEK293 cells from roller bottles were collected by centrifugation (1000*g*, 15 min), resuspended in 500 mM NaCl, 2.5mM $MgCl_2$, 50 mM Tris, pH 8.5, and lysed by three freeze/thaw cycles (alternating an ethanol/dry ice bath and a 37°C water bath). The cell lysate was clarified by centrifugation (8000*g*, 15 min). The pH of the clarified cell lysate was approximately 8.0. The supernatant was then diluted to 200 mM NaCl by the addition of sterile water and digested with Benzonase (purity Grade 1, Merck, Darmstadt, Germany; 200 U/mL, 1 h, 37°C). The lysate was adjusted to 25 mM $CaCl_2$ using a 1 M stock solution and incubated (1 h, 4°C). The mixture was centrifuged (8000*g*, 15 min) and the supernatant containing vector collected. To precipitate virus from the clarified cell lysate, polyethylene glycol (PEG 8000) was added to a final concentration of 8%, and the mixture was incubated (3 h, 4°C) and then centrifuged (8000*g*, 15 min). The pellets containing vector were resuspended with mixing in 0.15 M NaCl, 50 mM HEPES, 25 mM EDTA, pH 8.0, and incubated (16 h, 4°C). The resuspended material was pooled, and dry CsCl was added to a final density of 1.40 gm/mL. Vector was then banded by ultracentrifugation (SW28, 25,000 rpm, 24 h, 20°C) using a Beckman Model LE-80 centrifuge. The centrifugation tubes were fractionated, and densities from 1.38 to 1.42 gm/mL containing vector were pooled. The material was banded a second time by ultracentrifugation (NVT65 Rotor, 60,000 rpm, 16 h, 20°C), and fractions containing AAV2 vectors were pooled. Ultrafiltration/diafiltration (UF/DF) by tangential-flow filtration was used to achieve concentration and buffer exchange as described in the following section. In purification Method 2, cell harvests containing AAV were microfluidized and filtered through 0.65-and 0.2-μm filters (low protein binding, Sartorius, Goettingen, Germany). Virus was purified from the clarified cell lysates by chromatography using Poros 50HS cation-exchange resin (PE Biosystems, Foster City, CA, USA) as previously described [24]. For the nuclease digestion described in Fig. 2, column-purified vectors were incubated (4 h, RT) with 100 U/mL Benzonase and 10 U/mL DNase I (RNase free, Roche Applied Science, Penzberg, Germany). For purification Method 3, vectors obtained following chromatography were further purified by CsCl gradient ultracentrifugation (SW28, 25,000 rpm, 24 h, 20°C) to remove empty capsids.

Real-time quantitative PCR was used to quantify AAV preparations as previously described [25]. Vectors purified by each method were assessed by SDS–PAGE/silver staining analysis, and in all cases VP1, VP2, and VP3 were present in the expected ratios, with the capsid proteins representing >95% of total proteins as determined by scanning densitometry. However, unlike gradient-purified AAV2 vectors purified using Methods 1 and 3, vectors purified by Method 2 (column chromatography) contained empty capsids at a level ranging from 3 to 10 empty capsids per vector genome. We previously reported characterization of the quantity and size distribution of DNA impurities in AAV2 vectors prepared by these methods [22] (Smith et al., manuscript in preparation). The levels of residual plasmid and mammalian DNA were 11 and 35 $pg/10^9$ vg, respectively, for CsCl gradient-purified vector preparations, and 31 and 100 $pg/10^9$ vg, respectively, for column-purified vector. The higher level of DNA impurities in vectors purified by column chromatography corresponded to fragments of nonvector nucleic acids associated with empty/partially filled capsids that copurified with vector particles in the absence of a density gradient separation step. To measure the level of vector surface nucleic acid impurities, Benzonase digestion was performed by the mixing of 100 μL of purified vector with 400 μL of digestion buffer (10mM Tris, pH 8.0, 10mM $MgCl_2$, 500U Benzonase) and then incubation (60 min, 37°C). Within the limit of precision of the Q-PCR assay used (RSD ~10%), no significant reduction in plasmid and genomic DNA was observed following this nuclease treatment of column-or cesium gradient-purified vectors. In spike controls in which plasmid (250 pg) or genomic (85 ng) DNA was added to the vector, spiked DNA was fully digested. Southern blot analysis of the nonvector plasmid and mammalian DNA indicated a range of sizes up to the packaging limit of the vector (not shown).

Ultrafiltration/diafiltration. Disposable hollow-fiber tangential-flow filtration devices (8-in. Midgee, 100 kDa MW cutoff, Amersham Biosciences, Uppsala, Sweden) were used to concentrate and diafilter AAV2 vectors purified by the methods described above and for the UF/DF experiments described in Table 2. For all UF/DF procedures, a volume of diafiltration buffer corresponding to 10× the product volume was used, which was added in ~1-mL increments to approximate continuous diafiltration.

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Using this method, the calculated amount of residual CsCl after diafiltration was <0.5 mM. The following three formulations were used for UF/DF: Control Formulation (CF; 140 mM NaCl, 10 mM sodium phosphate, 5% sorbitol, pH 7.3, 592 mOsm); Test Formulation 1 (TF1; 150 mM sodium phosphate, pH 7.5, 355 mOsm); and Test Formulation 2 (TF2; 100 mM sodium citrate, 10 mM Tris, pH 8.0, 315 mOsm). For Experiment 1 shown in Table 2, diafiltration was performed at a volume corresponding to a concentration of 1×10^{13} vg/mL and then reduced to a value corresponding to 2.5×10^{13} vg/mL (assumes no vector loss). For Experiment 2, diafiltration was performed at a volume corresponding to a 2×10^{13} vg/mL and then reduced to a value corresponding to 6.7×10^{13} vg/mL. For Experiment 3 (CF ± Bz), AAV2-AADC vector (approximately 1.2×10^{14} vg) was first diafiltered into TF1 and then passed through a 0.2-μm filter. The titer was determined and the volume adjusted to 1×10^{13} vg/mL. To 10 mL of this material, $MgCl_2$ was added to a final concentration of 2 mM, and the material was then divided into two 5-mL aliquots. One aliquot was incubated with Benzonase (200 U/mL, 4 h, RT), and the second was mock-incubated. Each aliquot was then diafiltered into CF at a vector concentration of 2×10^{13} vg/mL and then concentrated to a 3.6×10^{13}-vg/mL target. Following all UF/DF protocols, Pluronic F-68 (BASF, Mount Olive, NJ, USA) was added to the vector product from a 1% stock to a final concentration of 0.001%, and the solution was passed through a 0.2-μm syringe filter (Sartorius).

Measurement of vector aggregation by dynamic light scattering. Purified vectors were assessed for aggregation by dynamic light scattering using Protein Solutions *DynaPro 99* (λ = 825.4 nm). Primary data (particle radius *Rh*, average value measured over 30 cycles, 10 cycles/min) were used for all analyses reported. A dilution-stress method was developed to assess the effect of varying excipients on vector aggregation. In this method, 80 μL of test excipient was added to 20 μL of purified vector with rapid mixing in the cuvette used for DLS measurement, and data collection was initiated within 10 s of mixing. Prior to the addition of excipients, the *Rh* value for AAV2 vector preparations was measured and confirmed to be <15 nm. Samples that were not 100% monomeric were passed through a 0.2-μm syringe disc filter (Sartorius) to remove aggregates. The osmolarity and ionic strength values shown in Fig. 1 were weighted calculations of test excipients (80%) and the starting vector formulations (20%). The osmolarity was calculated according to the equation: osmolarity = Σc_i, where c_i is the molar concentration of each solute species. The ionic strength (μ) was calculated according to the equation: $\mu = 1/2\Sigma c_i z_i^2$, where z_i is the charge on each species. Under conditions that resulted in vector aggregation (e.g., low μ), a progressive increase in *Rh* was observed over time. To validate the use of average *Rh* measured over the 3-min interval following dilution as a measure of aggregation, the average rate of increase of *Rh* ($\Delta Rh/\Delta t$) over the same time interval was also assessed, giving conclusions that were concordant with those reported in Fig. 1.

Functional studies. The infectivity of AAV2-AADC vectors was determined using a sensitive assay as previously described [15]. The test sample was run concurrently with an AAV2-AADC reference previously prepared in CF and stored at −80°C. The transduction efficiency of AAV2 vectors was measured using a whole-cell ELISA. Briefly, D7/4 cells grown in 96-well plates were infected with 10-fold serial dilutions of the test sample and reference vector at inputs ranging from 10 to 10^5 vg/cell (five replicates/dilution). After 48 h, the culture medium was removed, and cells were washed twice with 200 μL PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.2). Cells were then permeabilized and fixed by the addition of 100 μL PBS containing 0.5% Triton X-100 and 4% paraformaldehyde to each well (15 min). Cells were then washed twice with PBS containing 0.5% Triton X-100. Nonspecific sites were blocked by adding PBS containing 3% bovine serum albumin (BSA) and 0.5% Triton X-100 (60 min). After washing, cells were incubated (60 min) with rabbit anti-AADC IgG antibody (AB136, Chemicon, Temecula, CA, USA) and washed. Cells were then incubated (60 min) with alkaline phosphatase-conjugated goat anti-rabbit IgG and washed. Antibodies were diluted 1:1000 in PBS containing 1% BSA, 0.5% Triton X-100. The substrate para-nitrophenylphosphate (PNPP, Pierce Biotechnology, Rockford, IL, USA) was added (1 mg/mL in diethanolamine buffer, Pierce), and after 30 min the concentration of cleaved substrate was measured spectrophotometrically (λ = 405 nm). Human AADC expression as a function of vector input was fitted using a spline curve (SigmaPlot9.0, Systat Software, Point Richmond, CA, USA). The AAV2-AADC reference vector was measured concurrently with the test sample.

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Exhibit M

1. COMPONENTS OF THE DRUG PRODUCT [SRP-9001, SOLUTION FOR INFUSION]

1.1. Drug Substance

The drug substance (delandistrogene moxeparvovec) is diluted with formulation buffer containing excipients Sodium chloride, Tromethamine (Trometamol), Tromethamine HCl (Trometamol HCl), Magnesium chloride (Magnesium chloride hexahydrate) and Poloxamer 188, in order to prepare the drug product. The drug substance [redacted]

1.2. Excipients

The drug product is formulated in 7 mM Tromethamine (Trometamol) / 13 mM Tromethamine HCl (Trometamol HCl), 200 mM Sodium chloride, 1 mM Magnesium chloride (Magnesium chloride hexahydrate), 0.001% Poloxamer 188, at pH 8.0 ± 0.5, and is the same formulation as the drug substance. The components of the formulation are commonly used as ingredients of intravenous formulations and were selected to provide stability and compatibility of the drug product for the intended route of administration.

The extent of AAV aggregation is a critical concern for safety and efficacy in a drug product. It has been reported in the literature that ionic strength is critical for the minimization of AAV aggregation (Wright 2005). [redacted]

7 mM Tromethamine (Trometamol) / 13 mM Tromethamine HCl (Trometamol HCl) was selected as a buffering agent [redacted]

A neutral pH has also been shown to minimize freeze-thaw induced AAV aggregation (as compared to acidic pH) (Croyle 2001).

Non-ionic surfactants such as Poloxamer 188 have been shown to be effective in recovery of AAVs under different conditions (Bennicelli 2008). [redacted]

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Exhibit N

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2. DRUG PRODUCT [SRP-9001, SOLUTION FOR INFUSION]

2.1. Formulation Development

Development of SRP-9001 drug product formulation was based on available knowledge of physicochemical properties of recombinant adeno-associated virus (AAV) serotypes. While structure of different AAV serotypes are known to vary, significantly impacting their mechanisms of cellular entry and trafficking, their biophysical properties that are key to developing formulation strategies are applicable for a broad range of AAVs.

The SRP-9001 drug product is a sterile solution for intravenous administration. The product is manufactured and stored as a sterile frozen liquid formulation and packaged in 10 mL sterile Crystal Zenith (CZ, cyclic olefin polymer) vials.

The drug product contains 1.33×10^{13} vg/mL of delandistrogene moxeparvovec formulated in 7 mM Tromethamine (Trometamol), 13 mM Tromethamine HCL (Trometamol HCl) (also known as 20 mM Tris), 200 mM Sodium chloride, 1 mM Magnesium chloride (Magnesium chloride hexahydrate), 0.001% Poloxamer 188, at pH 8.0 ± 0.5. The components of the formulation are commonly used as ingredients of intravenous formulations and were selected to provide stability and compatibility of the drug product for the intended route of administration.

The pH, osmolality, and the extent of aggregation are critical for the safety and efficacy of intravenous infusion products which are not diluted, such as SRP-9001. It has been reported in the literature that ionic strength is critical to reduce aggregation of AAVs (Wright 2005). It has been further shown that the inhibition of AAV aggregation by salt was correlated to ionic strength and not necessarily to osmolality. [redacted] A neutral pH has also been shown to minimize freeze-thaw induced AAV aggregation (as compared to acidic pH) (Croyle 2001).

Non-ionic surfactants such as Poloxamer 188 have been shown to be effective in recovery of AAVs under different conditions (Bennicelli 2008). [redacted]

The same formulation has been used from initial clinical trials through to the commercial product. Use of these excipients and their levels in SRP-9001 drug product is supported by studies reported in literature, formulation robustness studies (Section 2.1.1) as well as ongoing stability studies (see Section 3.2.P.8.1).

2.1.1. Formulation Robustness Studies

[redacted]

[redacted]

2.1.1.1. Description of the Analytical Methods Used

2.1.1.1.1. Dynamic Light Scattering (DLS)

Vector aggregation was measured using Dynamic Light Scattering (DLS) on a Zetasizer Ultra. A low volume disposable sizing cell which can accommodate from 3 to 30µL of sample was used for the DLS measurements. A disposable capillary was loaded with about 10 µL of the sample by the capillary action method. The capillary was sealed at both ends using sealing clay. Refractive index for the dispersant was set at [redacted] and viscosity at [redacted] Sample was equilibrated for [redacted] seconds before taking the measurements. A general-purpose model was selected for data analysis. An average of 3 readings per sample were recorded for Z-average size and polydispersity index (PDI) along with the standard deviation.

2.1.1.1.2. Subvisible Particle Analysis by Microflow Imaging

Microflow imaging (MFI) analysis was performed using a 400 µm wide and 1.6 cm long Silane coated flow cell. A 5 µm size and count standard was run to confirm instrument performance both prior to and after sample measurements. Blank measurements were run between each sample to ensure cleanliness and no particle carry-over between runs. Depending on the sample availability, SRP-9001 DP samples were either diluted [redacted] for analysis or used as-is without dilution. A total of 0.95 mL sample was injected per analysis out of which [redacted] was used for data analysis with a purge volume of [redacted]

2.1.1.1.3. Differential Scanning Fluorimetry by UNcle

Capsid stability was analyzed by measuring the differential scanning fluorescence. This assay is based on the principle that SYBR Gold dye upon binding to DNA can undergo fluorescence emission. The sample was heated from 25°C to 95°C with a [redacted] ramp rate. The DNA ejection test method allowed for the detection of extraneous DNA by binding it to SYBR Gold dye, both at its original state- known as initial or baseline fluorescence (InFl), and when heated over a temperature gradient from 25°C to 95°C. A % increase in InFl was calculated by measuring an increase in InFl of the sample with respect to the negative control (formulation buffer + dye) during the analysis. A final fluorescence (FF) measurement was also obtained again at [redacted] Additionally, the DNA ejection temperature (T_{m1}) and capsid melting temperature (T_{m2}) were reported. Static light scattering at 473 nm was used to measure the aggregation temperature of the capsid, T_{agg}.

2.1.1.1.4. Aggregation by Size Exclusion Chromatography-Multi Angle Light Scattering

SRP-9001 samples were evaluated for aggregates using size exclusion chromatography equipped with UV, multi-angle light scattering and a refractive index detector. Briefly, [redacted] of freshly thawed drug product sample was injected on a size exclusion chromatography column (3.5 micron, 7.8 × 300 mm). Uncertainty-weighted average radius of monomer peak was obtained for each sample. The area percent of DNA peak, dimer peak and monomer peak was determined using relative UV peak area at [redacted]

2.1.1.1.5. Capsid Titer by Octet

Viral capsid titer was measured by Bio-Layer Interferometry. This assay is based on the principle where the binding rate of AAV to AAVX bound to octet biosensor is measured (Biotinylated AAVX is bound to streptavidin precoated octet biosensor). The assay consists of 4 steps: 1) obtaining baseline signal of streptavidin coated biosensor with sample diluent buffer, 2) binding of biotinylated AAVX ligand to the streptavidin biosensor, 3) re-measuring the baseline signal, and 4) AAV binding to the AAVX. Standard curve is generated using known AAV concentration and the binding rate, which is used to calculate the final AAV capsid titer using the binding rate obtained for the samples.

2.1.1.1.6. Vector Genome Titer by ddPCR

Viral genome titer was measured using ddPCR. Samples are initially treated with DNase to remove any non-encapsidated DNA, [redacted] The ddPCR is performed on this released DNA using primers specific to the MHCK7 promoter, the procedure contains 3 steps, 1) reaction partitioning 2) target amplification and 3) droplet reading. This assay is based on the principle that sample reactions are partitioned into approximately [redacted], and the viral genome is amplified within each droplet using MHCK7 targeting primer and fluorescently labeled probe. Each amplification will lead to activation of fluorophore, and each droplet is measured for positive or negative fluorophore signal which is quantified to copy number per µL concentration by instrument software with built in inherent Poisson equation based on the number of positive and negative fluorophore signals.

2.1.1.1.7. Capsid Purity by CE-SDS

Capsid purity was analyzed by capillary electrophoresis sodium dodecyl sulfate (CE-SDS). This assay relies on separation of SDS-labeled protein variants by a polymeric matrix in constant electrophoretic conditions. A280 protein concentration of the sample is measured using and diluted to [redacted] if required, followed by [redacted] dilution using sample buffer. The sample is then reduced to native protein form using the reducing agent beta-mercaptoethanol (BME) followed by incubation at [redacted] minutes. A fluorescent dye, that labels primary amine groups within proteins, was added to the sample after incubation, and incubated at [redacted] minutes. A color change from blue to red is exhibited indicating binding of dye to primary amines, the sample is further diluted 4-fold using UltraPure™ water and is analyzed using Laser-Induced Fluorescence (LIF) detection to obtain protein purity with excitation and emission wavelengths set at 488 and 600 nm respectively. [redacted]

2.1.1.2. Vg Titer Ranging Study

Effect of varying the physical titer in the drug product from [redacted] was studied at time zero and after 4 days of storage at 25°C, and the results are presented in Table 2. The sample formulations, designated as Formulation 1 through 4 were made by diluting the drug substance in Table 1 with formulation buffer to reach the intended vg titer over a range of [redacted]

[redacted]

Table 2: Stability of DP as a Function of Vg Titer

2.1.1.3. pH Ranging Study

Table 3: Stability of DP as a Function of pH

2.1.1.4. Sodium Chloride Ranging Study

Table 4: Stability of DP as a Function of Sodium Chloride Concentration

Figure 1: Z-Average from DLS for Sodium Chloride Ranging Studies

Figure 2: Percent Increase in Initial Fluorescence for Sodium Chloride Ranging Studies

Figure 3: Result on Purity by SDS-PAGE for NaCl Ranging Studies

2.1.1.5. Magnesium Chloride Ranging Study

Table 5: Stability of DP as a Function of Magnesium Chloride Concentration

Figure 4: Results of Percent Increase in Initial Fluorescence Over Magnesium Chloride Concentrations

Figure 5: Peak Areas for DNA, Dimer (A) and Monomer (B) as Determined by SEC over Magnesium Chloride Concentrations

(B)

Figure 6: Results on Purity by SDS-PAGE for Magnesium Chloride Ranging Study

2.1.1.6. Poloxamer-188 Ranging Study

Table 6: Stability of DP as a Function of Poloxamer Concentration

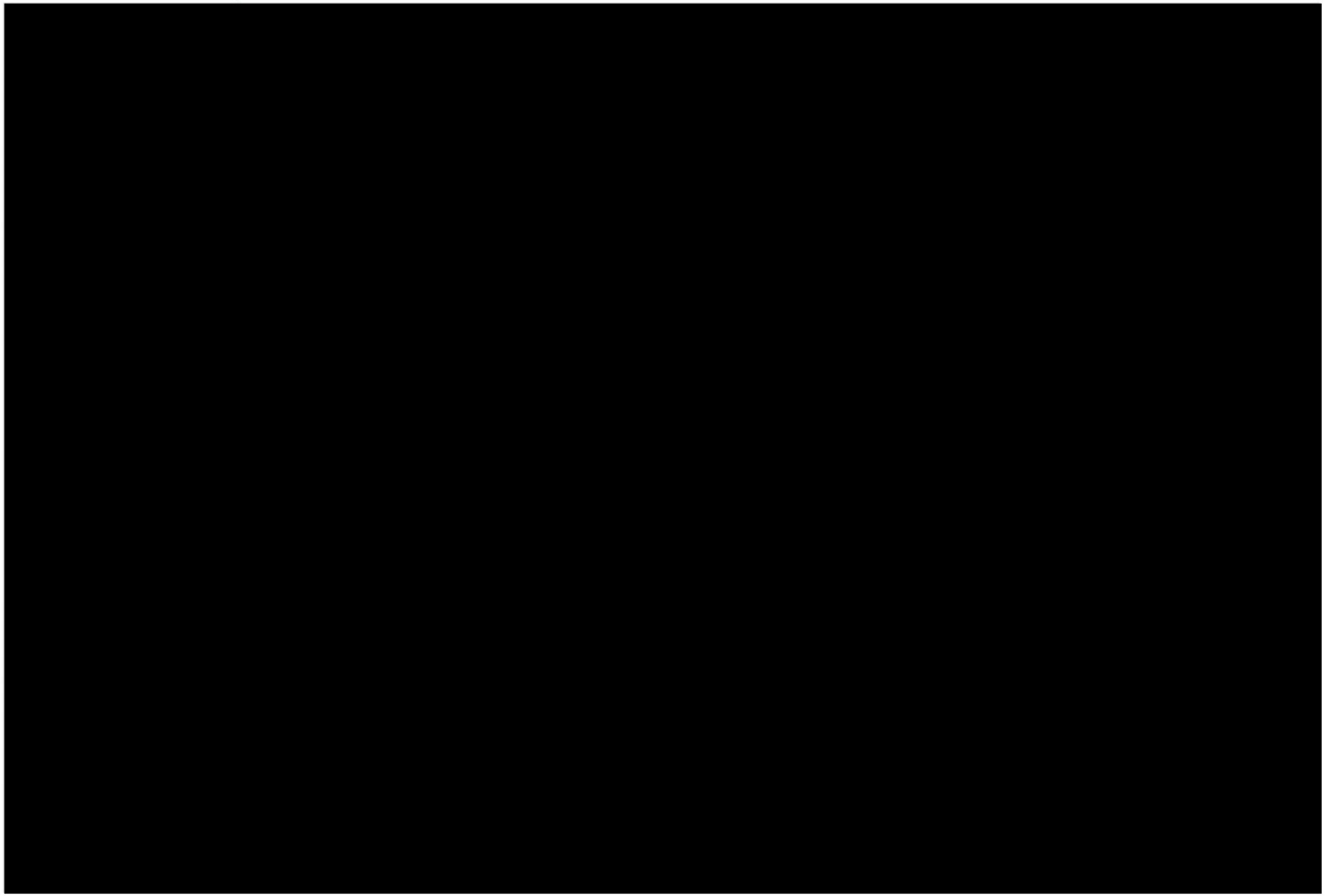


Table 7: Results for Stirred Samples of SRP-9001 DP With and Without Poloxamer

2.1.2. Long-Term Stability Storage Temperature for SRP-9001

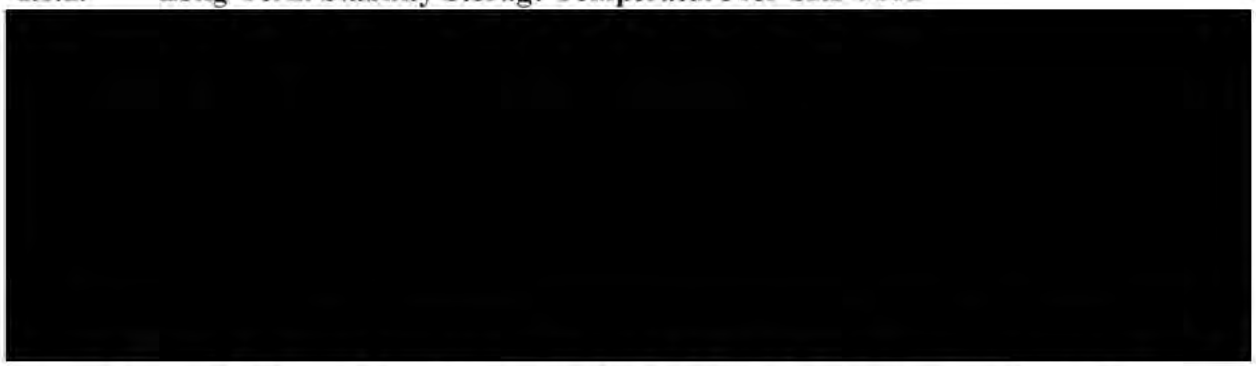


Figure 7: Cooling and heating profiles for SRP-9001 DP by differential scanning calorimetry

2.1.3. Characterization Discussion on Inherent Particles in SRP-9001

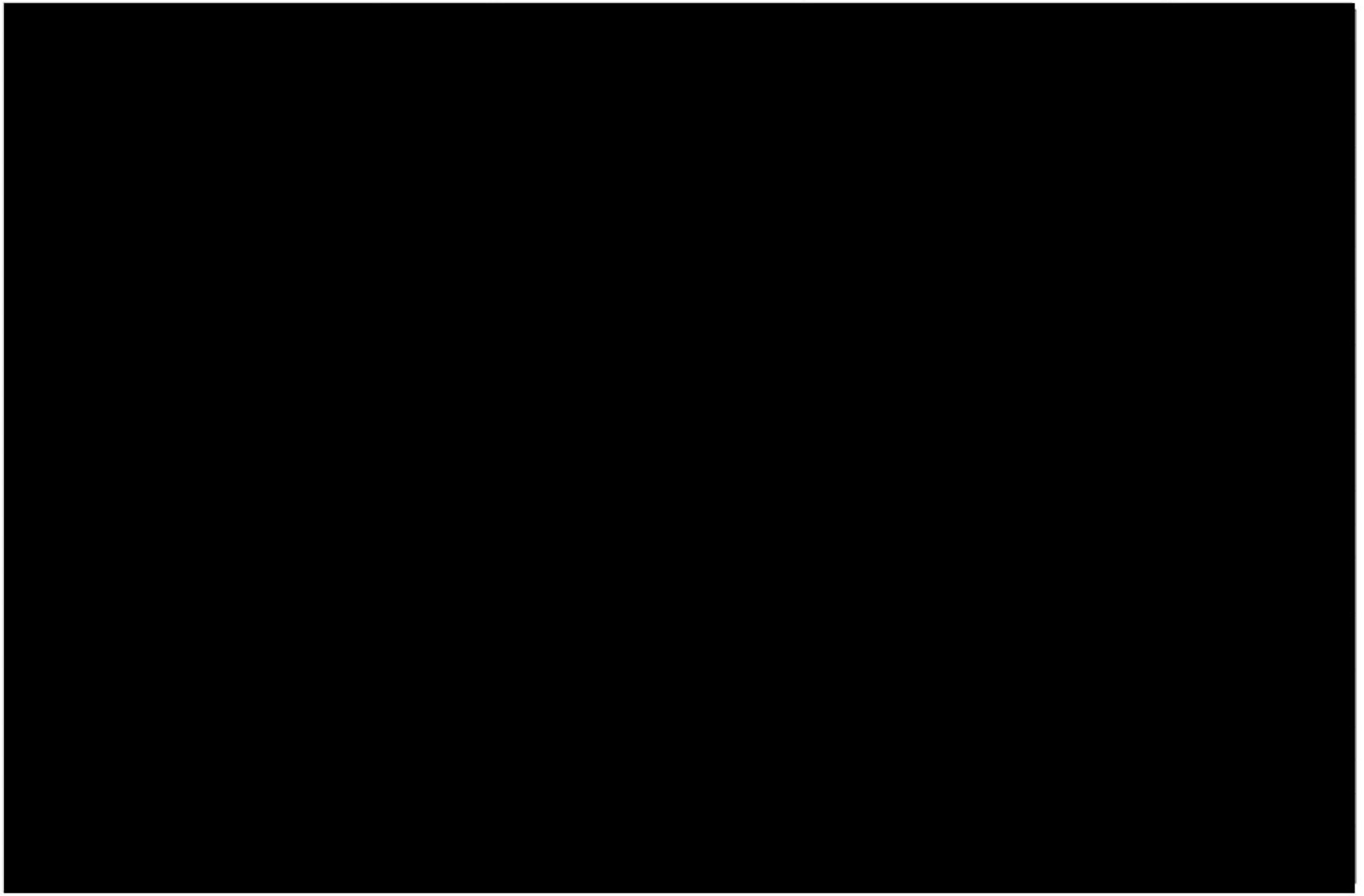
Figure 9: Example of Raman Spectrum of a Typical SRP-9001 Drug Product Particle and Corresponding Raman Spectrum of IgG (Control Protein)

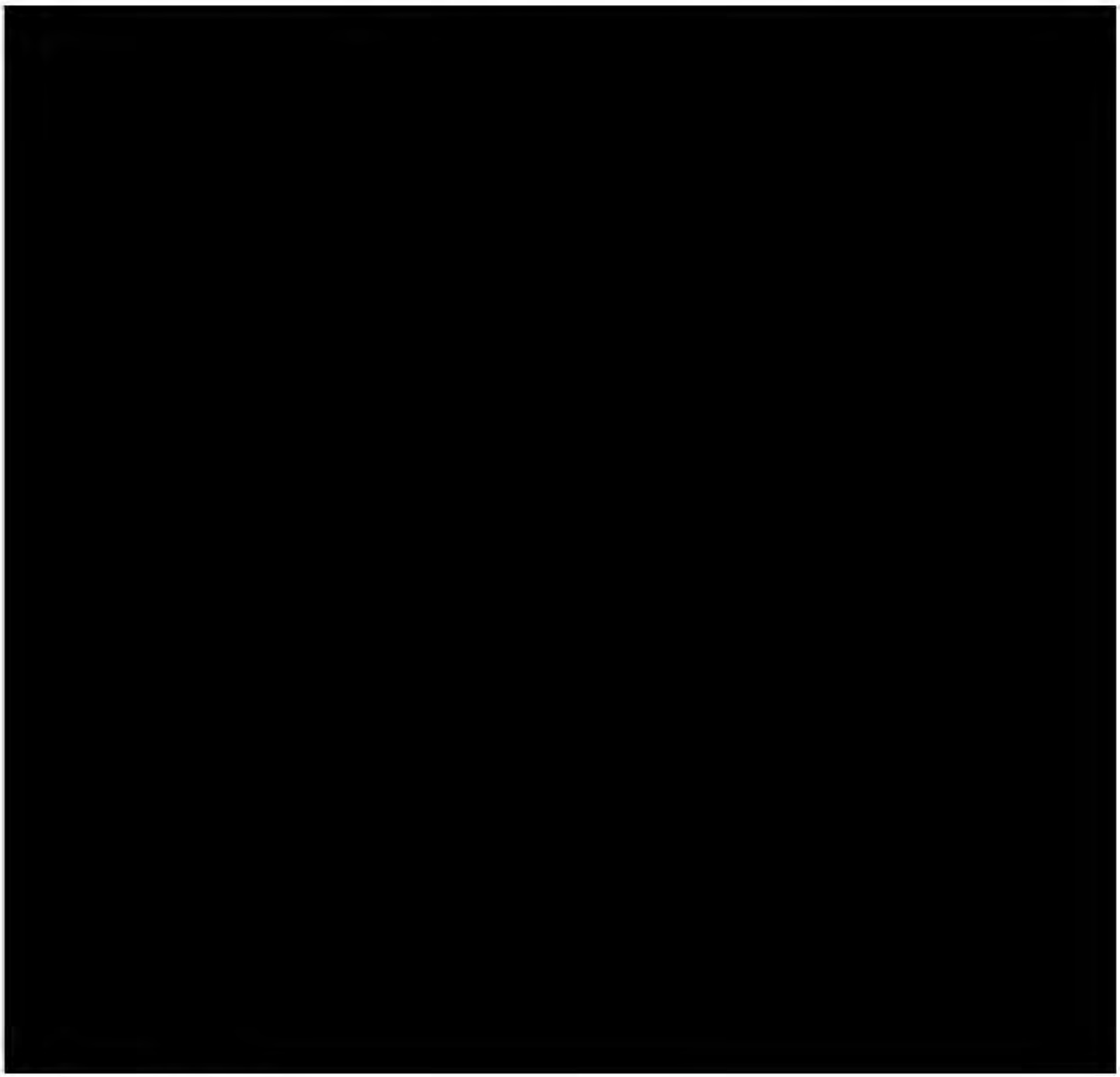
[redacted]

2.1.4. Effect of Agitation

[redacted]

Table 8: Results for All Analytical Assays Performed on Stirred Samples for SRP-9001 Over 96 hr of Agitation





2.2. Overages

2.3. Physicochemical and Biological Properties

The SRP-9001 drug product is a gene therapy medicinal product that expresses a human SRP-9001dystrophin protein. It is a non-replicating recombinant adeno-associated virus (rAAV) based vector containing cDNA encoding a shortened, but functional form of the human dystrophin gene under control of the α-myosin heavy-chain creatine kinase 7 (MHCK7) promoter to restrict expression to skeletal and cardiac muscle. The drug product is formulated in 7 mM Tromethamine (Tromethamol)/13 mM Tromethamine HCl (Trometamol HCl),

3.2.P.2. Pharmaceutical Development [SRP-9001, Solution for infusion]

200 Sodium Chloride, 1 mM Magnesium Chloride (Magnesium Chloride Hexahydrate), 0.001% Poloxamer 188, at pH 8.0.

[redacted]

Table 9: Summary of Solution Properties for Drug Product

[redacted]

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Exhibit O

US012031894B2

(12) **United States Patent**
O'Riordan et al.

(10) **Patent No.: US 12,031,894 B2**
(45) **Date of Patent: *Jul. 9, 2024**

(54) **ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES**

(71) Applicant: **Genzyme Corporation**, Cambridge, MA (US)

(72) Inventors: **Catherine R. O'Riordan**, Cambridge, MA (US); **Brenda Burnham**, Hopkinton, MA (US)

(73) Assignee: **GENZYME CORPORATION**, Cambridge, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **18/513,970**

(22) Filed: **Nov. 20, 2023**

(65) **Prior Publication Data**

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(60) Provisional application No. 62/105,714, filed on Jan. 20, 2015.

(51) **Int. Cl.**

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(52) **U.S. Cl.**

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(58) **Field of Classification Search**

CPC .. C07K 2317/92; C07K 14/005; A61K 39/12; C12N 15/86; C12N 2750/14143; C12N 15/864; C12N 2750/14152; G01N 33/68; G01N 2001/4083; C12Q 1/70

See application file for complete search history.

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(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 (fragmented 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.

30 Claims, 17 Drawing Sheets

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Petition for Inter Partes Review for U.S. Pat. No. 10,429,288, dated Jun. 30, 2023, Case No. IPR2023-1044, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 80 pages.

Petitioner Novartis Gene Therapies, Inc.'s Power of Attorney and Designation of Lead and Backup Counsel for U.S. Pat. No. 10,429,288, dated Jun. 20, 2023, Case No. IPR2023-1045, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 3 pages.

Petitioner Novartis Gene Therapies, Inc.'s Power of Attorney and Designation of Lead and Backup Counsel for U.S. Pat. No. 10,429,288, dated Jun. 20, 2023, Case No. IPR2023-1044, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 3 pages.

Petitioner Novartis Pharmaceuticals Corporation's Power of Attorney and Designation of Lead and Backup Counsel for U.S. Pat. No. 10,429,288, dated Jun. 20, 2023, Case No. IPR2023-1045, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 3 pages.

Petitioner Novartis Pharmaceuticals Corporation's Power of Attorney and Designation of Lead and Backup Counsel for U.S. Pat. No. 10,429,288, dated Jun. 20, 2023, Case No. IPR2023-1044, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 3 pages.

Petitioner's Exhibit List for U.S. Pat. No. 10,429,288, dated Jun. 30, 2023, Case No. IPR2023-1045, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 4 pages.

Petitioner's Exhibit List for U.S. Pat. No. 10,429,288, dated Jun. 30, 2023, Case No. IPR2023-1044, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 4 pages.

Petitioner's Supplemental Paper and Notice Ranking Petitions for U.S. Pat. No. 10,429,288, dated Jun. 30, 2023, Case No. IPR2023-1045, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 5 pages.

Petitioner's Supplemental Paper and Notice Ranking Petitions for U.S. Pat. No. 10,429,288, dated Jun. 30, 2023, Case No. IPR2023-1044, Petitioner Novartis Gene Therapies Inc. and Novartis Pharmaceuticals Corporation, Patent Owner Genzyme Corporation, 5 pages.

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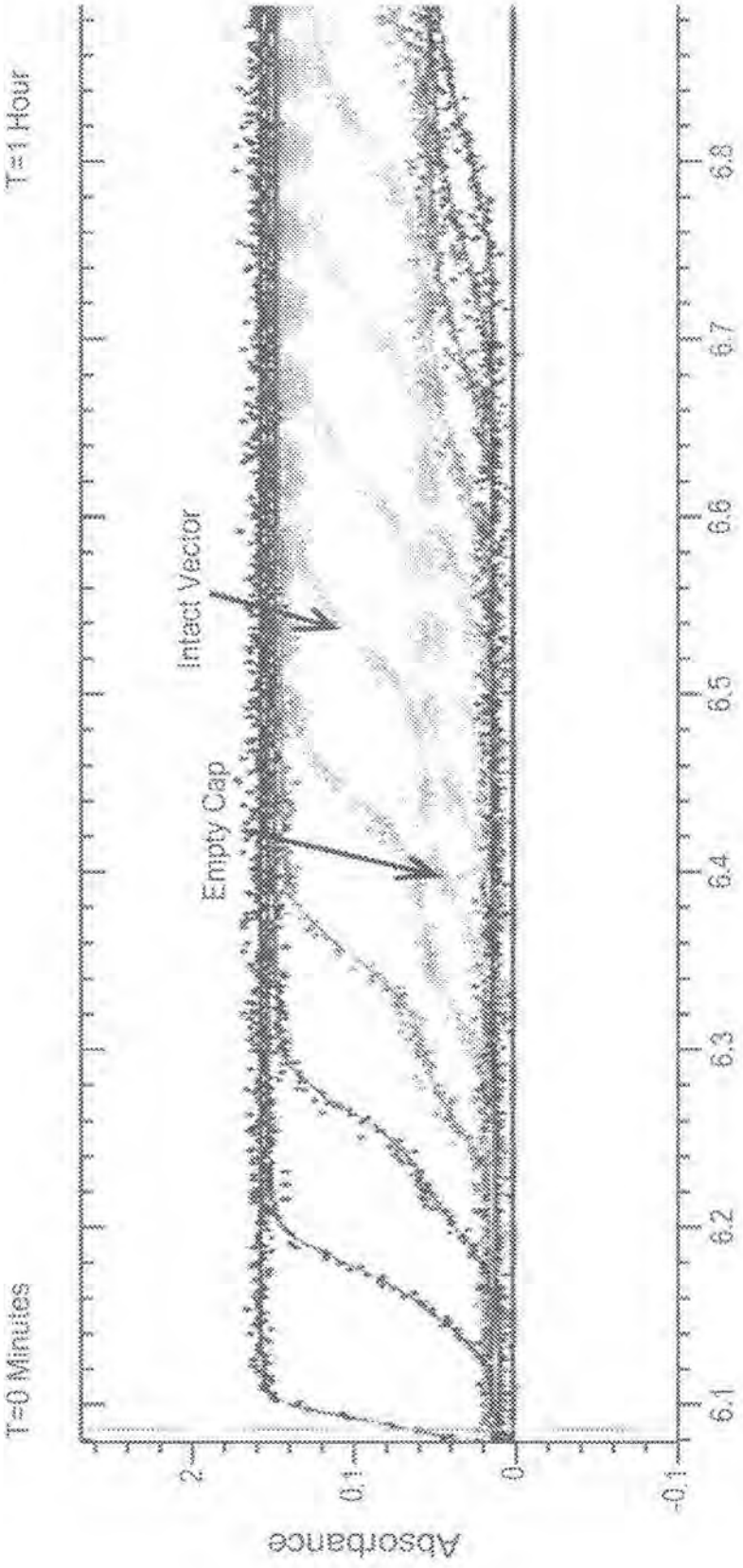

T=1 Hour
T=0 Minutes
Intact Vector
Empty Cap
Absorbance
2
0.1
0
-0.1
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
Radius, cm

FIG. 1A

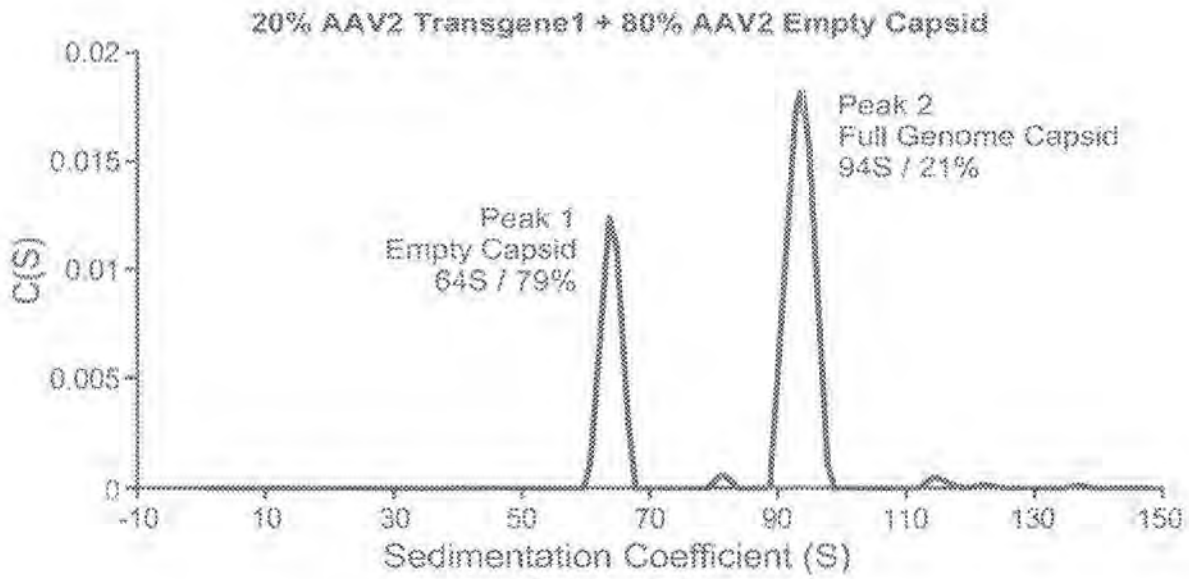

20% AAV2 Transgene1 + 80% AAV2 Empty Capsid
0.02
0.015
0.01
0.005
0
c(s)
Peak 1
Empty Capsid
64S / 79%
Peak 2
Full Genome Capsid
94S / 21%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 1B

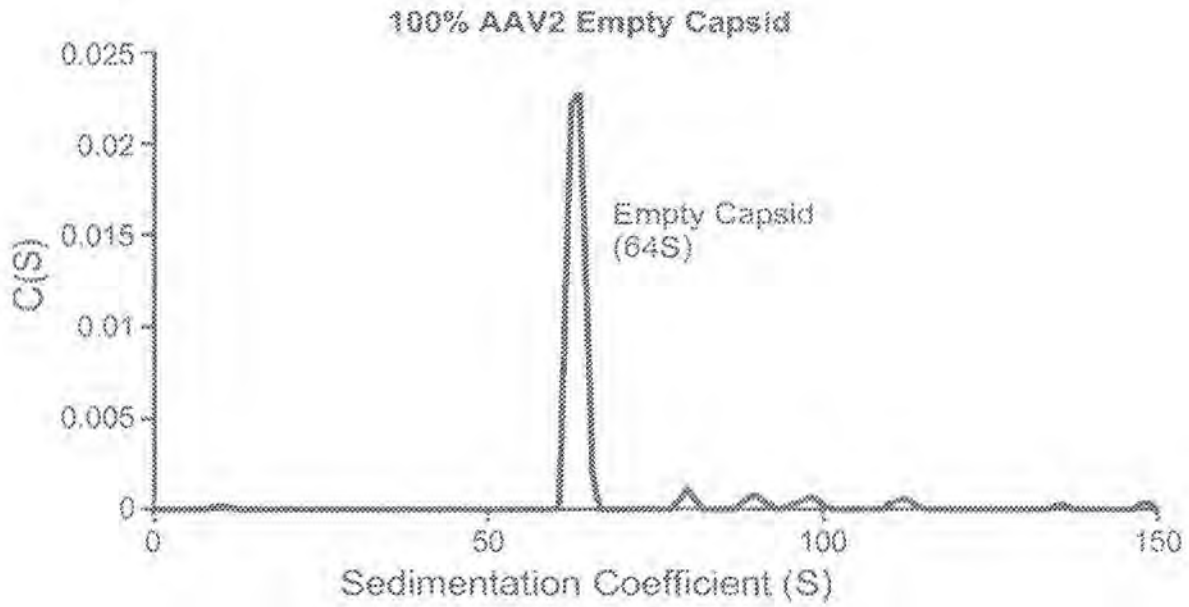

100% AAV2 Empty Capsid
0.025
0.02
0.015
0.01
0.005
0
C(S)
Empty Capsid
(64S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2A

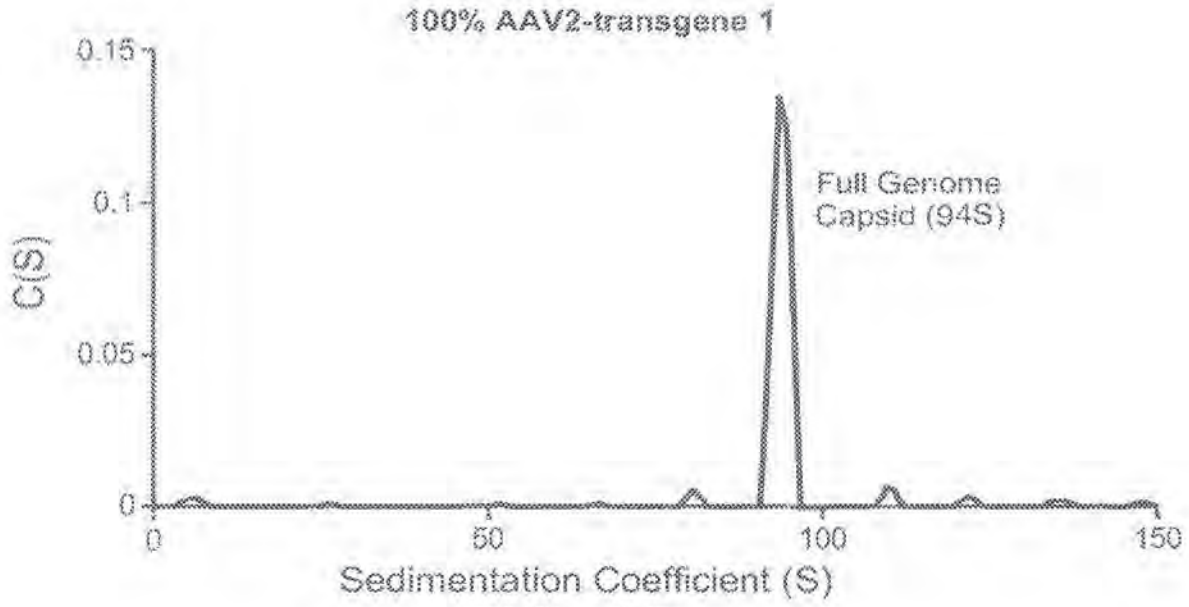

100% AAV2-transgene 1
0.15
0.1
0.05
0
C(S)
Full Genome
Capsid (94S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2B

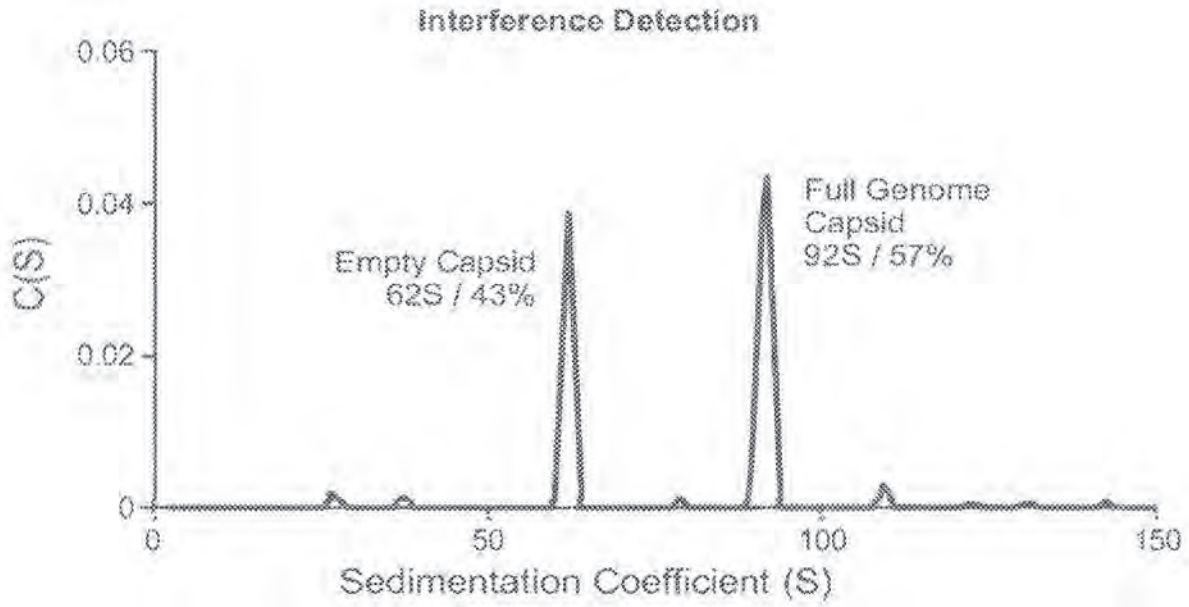

Interference Detection
0.06
0.04
0.02
0
C(S)
Empty Capsid
62S / 43%
Full Genome
Capsid
92S / 57%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3A

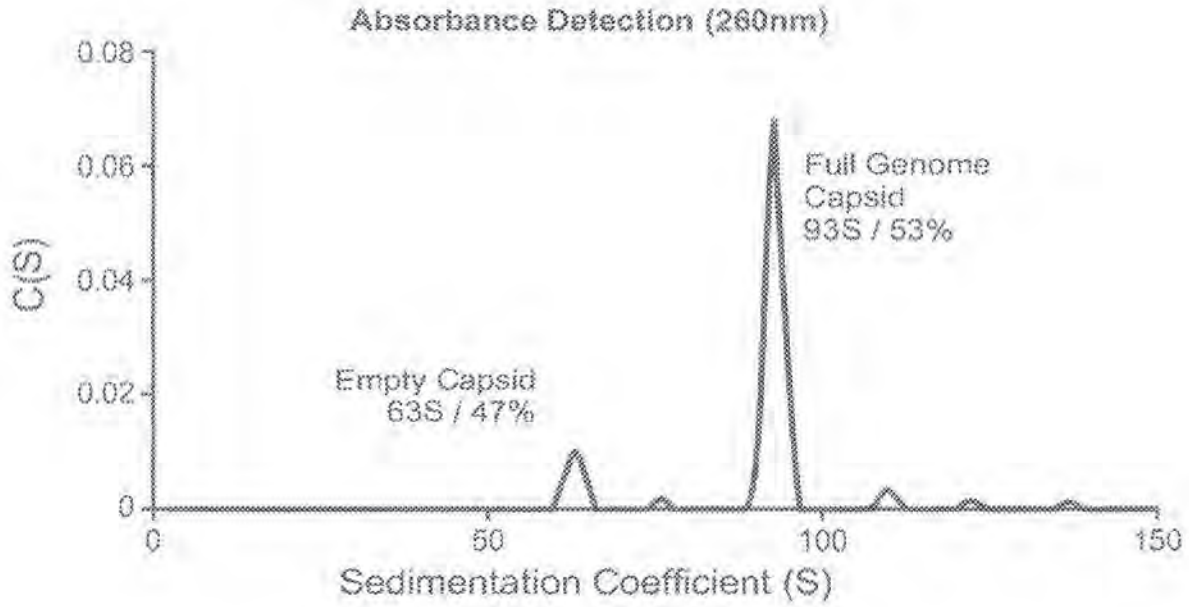

Absorbance Detection (260nm)
0.08
0.06
0.04
0.02
0
C(S)
Empty Capsid
63S / 47%
Full Genome
Capsid
93S / 53%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3B

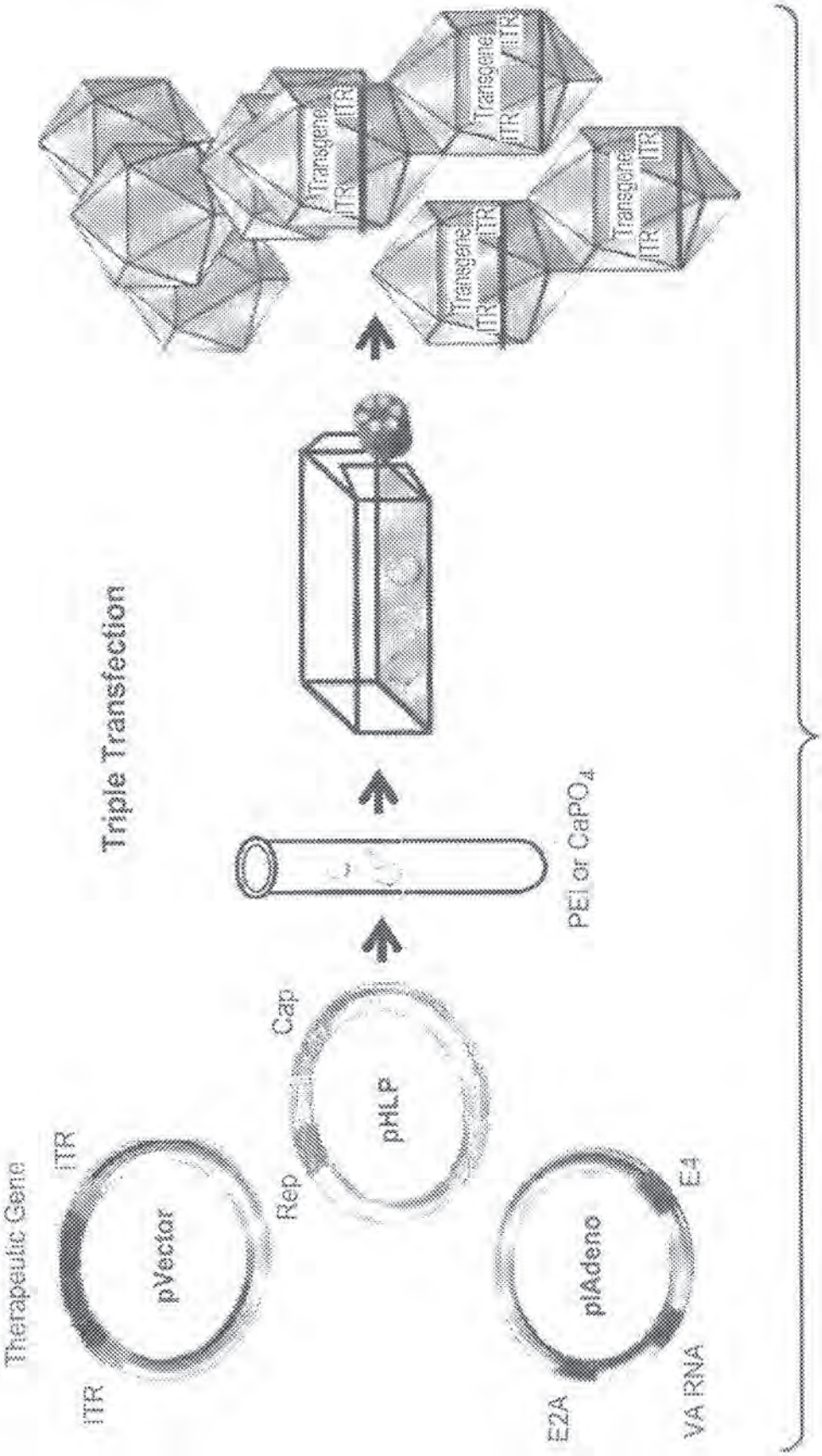

Transgene
ITR
Triple Transfection
PEI or $CaPO_4$
Cap
pHLP
Rep
Therapeutic Gene
ITR
pVector
ITR
pIAdeno
E4
E2A
VA RNA

FIG. 4

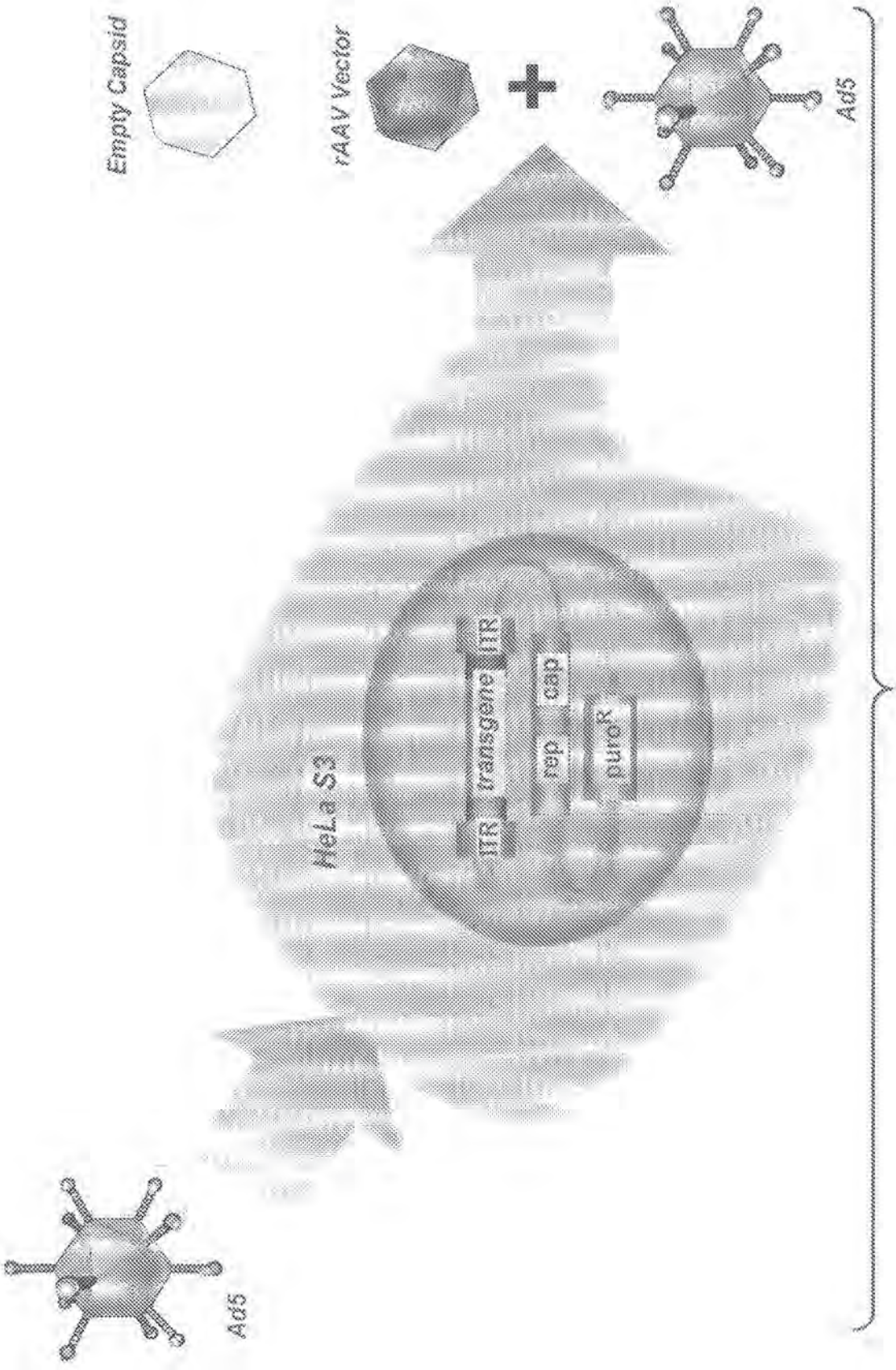
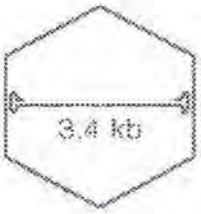

Empty Capsid
rAAV Vector
Ad5
HeLa S3
ITR
transgene
ITR
cap
rep
puroR
Ad5

FIG. 5

FIG. 6A



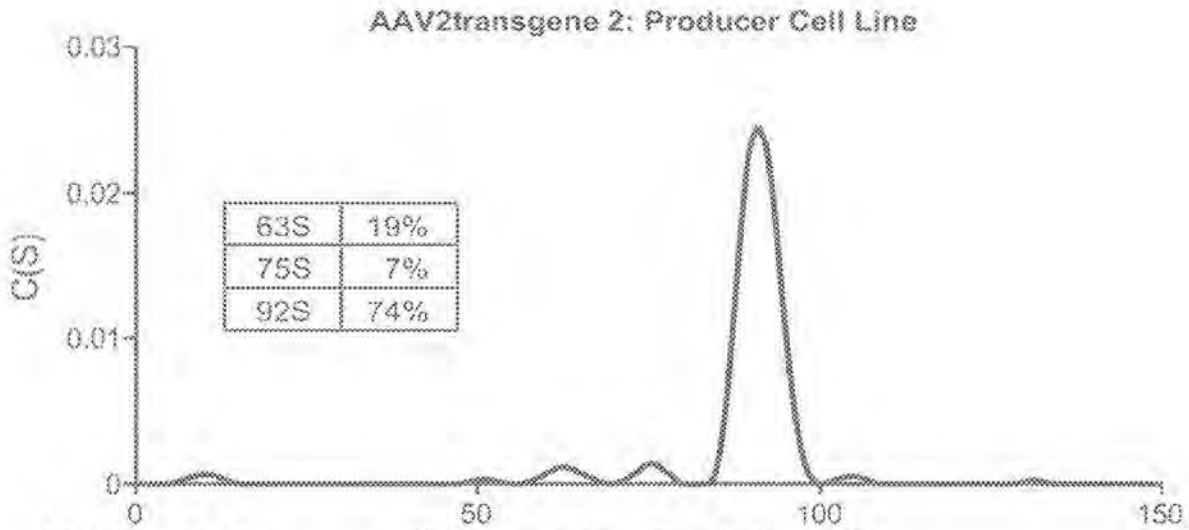


FIG. 6B

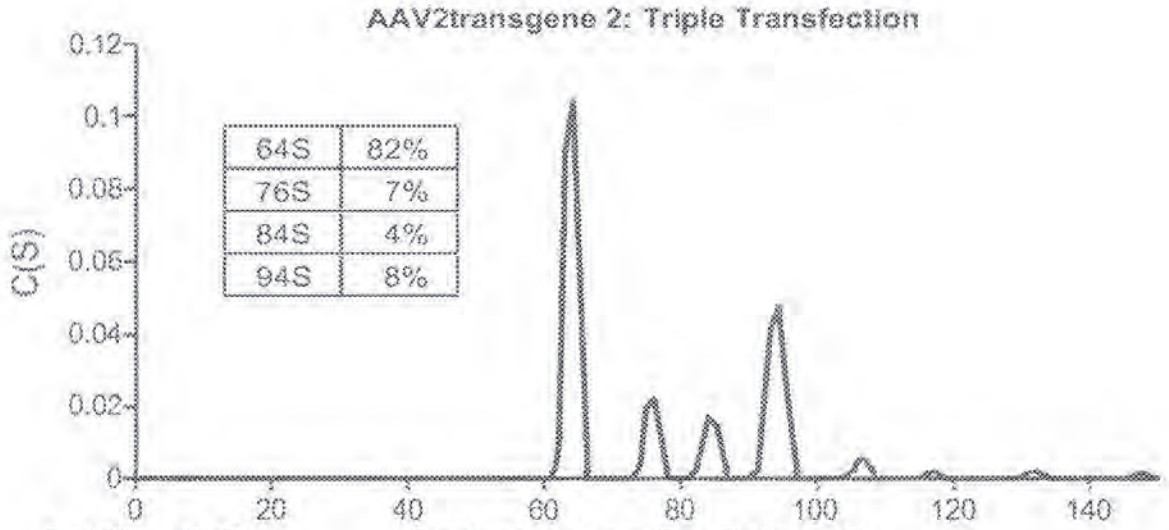


FIG. 6C

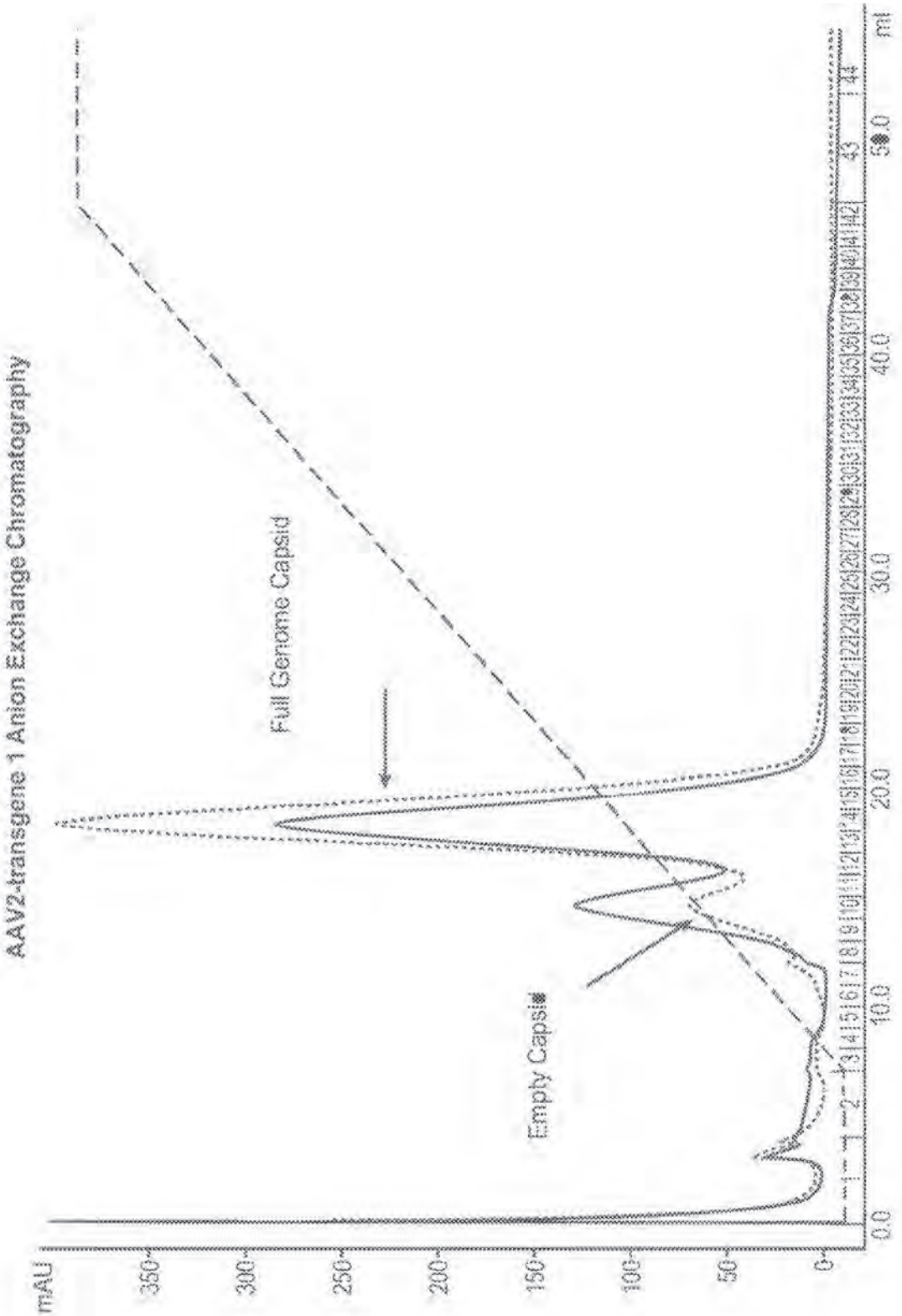

AAV2-transgene 1 Anion Exchange Chromatography
Full Genome Capsid
Empty Capsid
mAU
350
300
250
200
150
100
50
0
0.0
10.0
20.0
30.0
40.0
50.0
ml

FIG. 7A

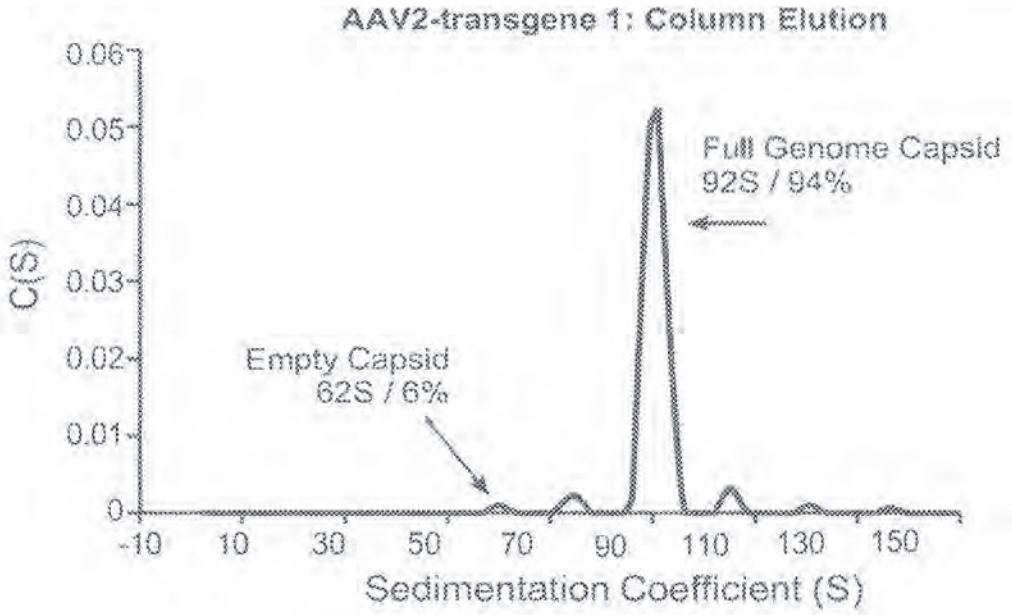

AAV2-transgene 1: Column Elution
0.06
0.05
0.04
0.03
0.02
0.01
0
C(S)
Full Genome Capsid
92S / 94%
Empty Capsid
62S / 6%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7B

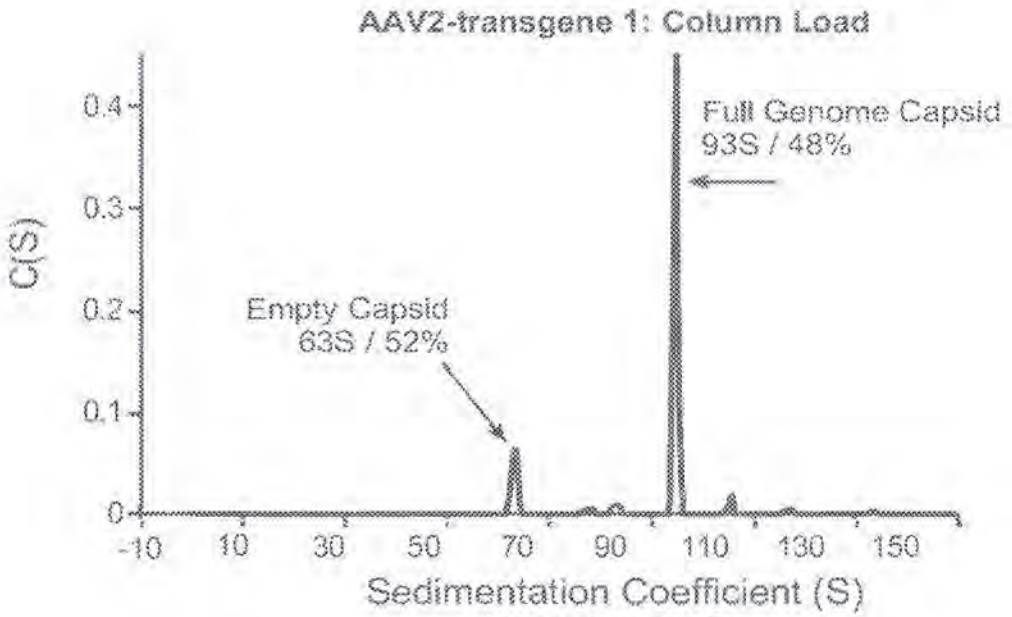

AAV2-transgene 1: Column Load
0.4
0.3
0.2
0.1
0
C(S)
Full Genome Capsid
93S / 48%
Empty Capsid
63S / 52%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7C

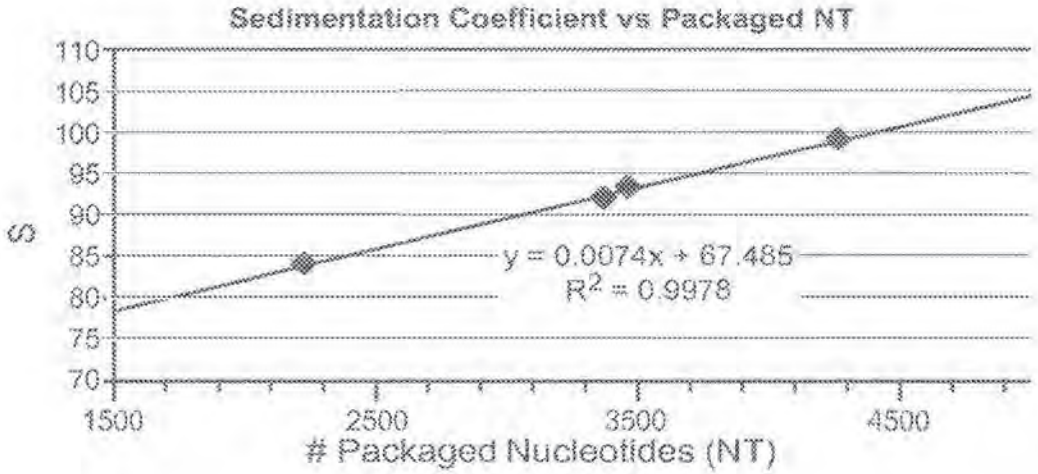

Sedimentation Coefficient vs Packaged NT
S
110
105
100
95
90
85
80
75
70
y = 0.0074x + 67.485
R2 = 0.9978
1500
2500
3500
4500
Packaged Nucleotides (NT)

FIG. 8

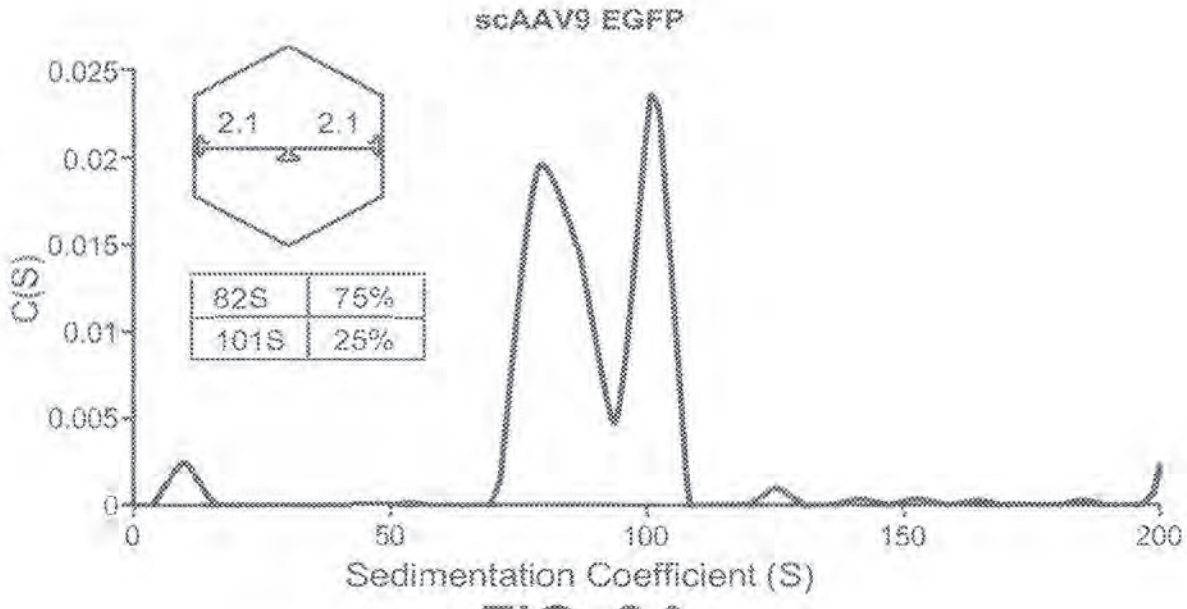

scAAV9 EGFP
C(S)
0.025
0.02
0.015
0.01
0.005
0
2.1
2.1
82S 75%
101S 25%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9A

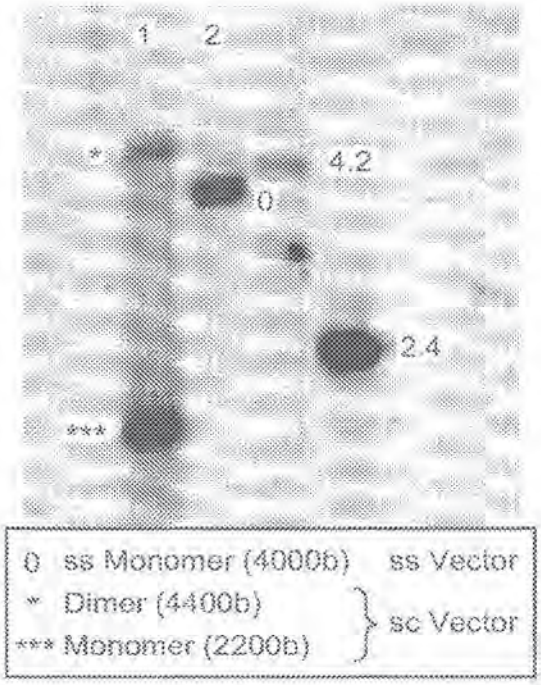

1
2
*
4.2
0
2.4
***
0 ss Monomer (4000b) ss Vector
* Dimer (4400b)
*** Monomer (2200b)
sc Vector

FIG. 9B

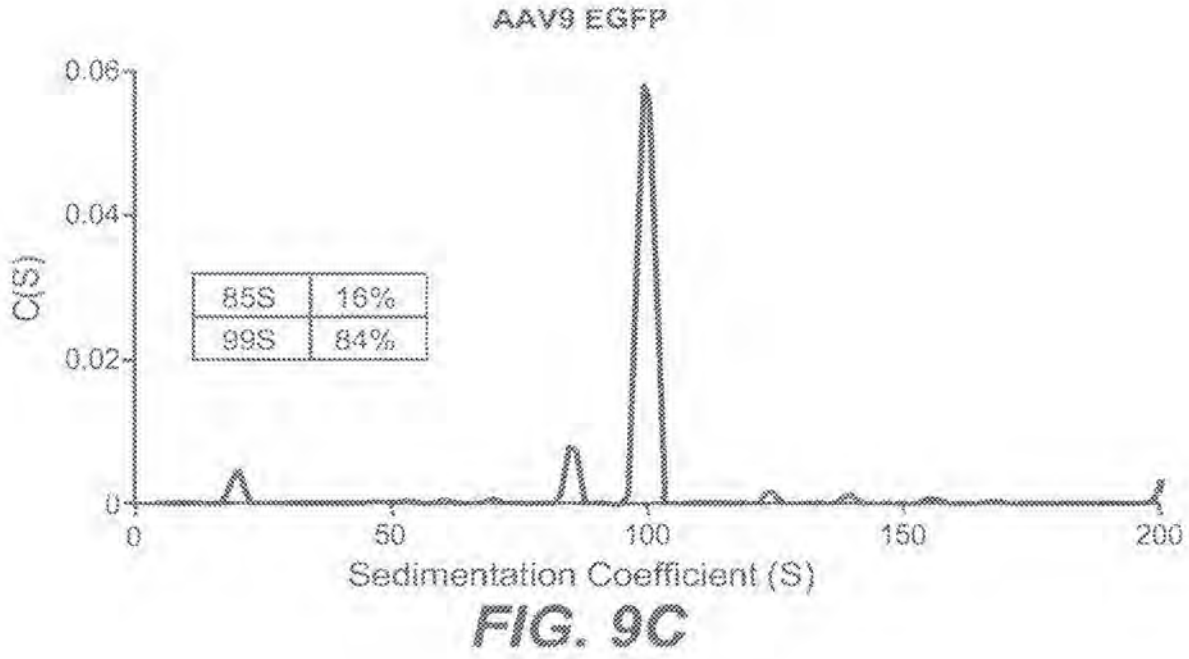

AAV9 EGFP
0.06
0.04
0.02
0
C(S)
85S 16%
99S 84%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9C

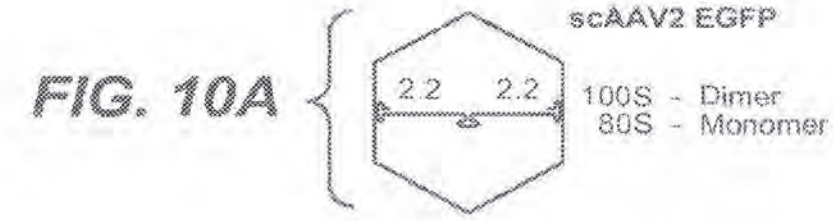


FIG. 10A

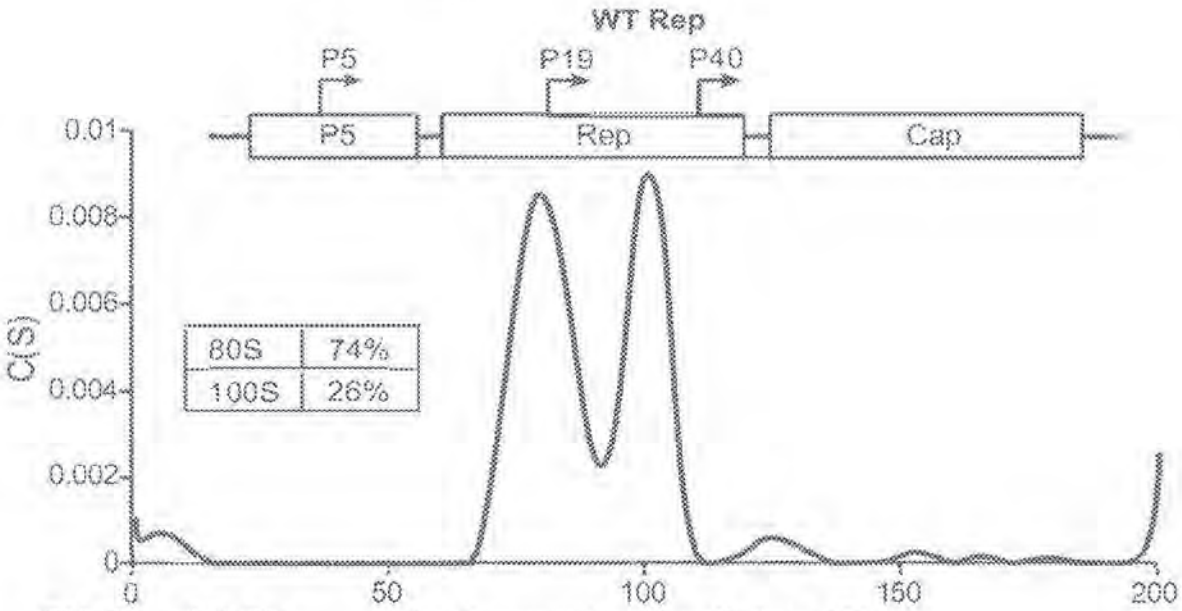


FIG. 10B

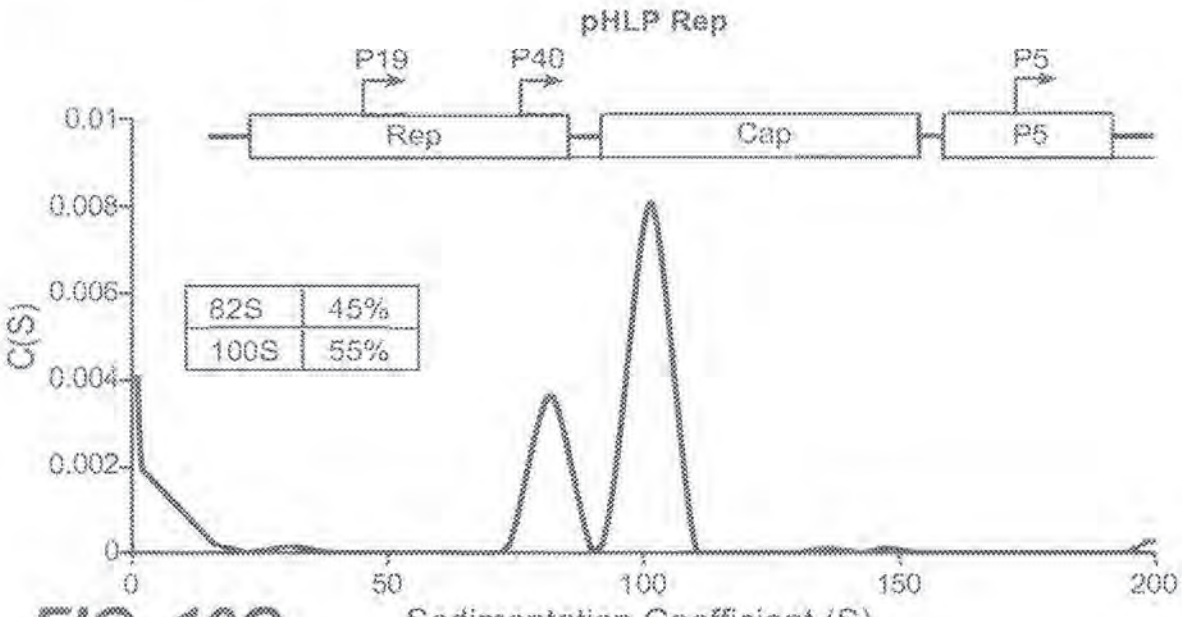


FIG. 10C

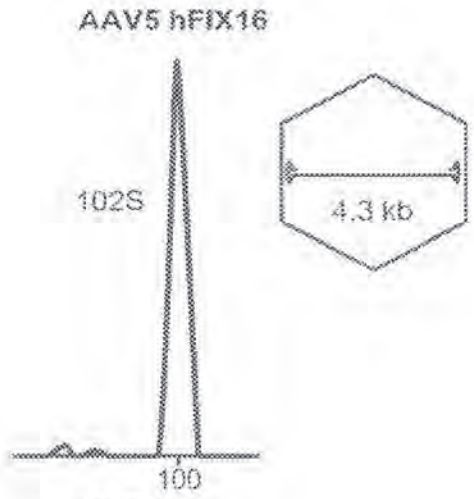


FIG. 11A

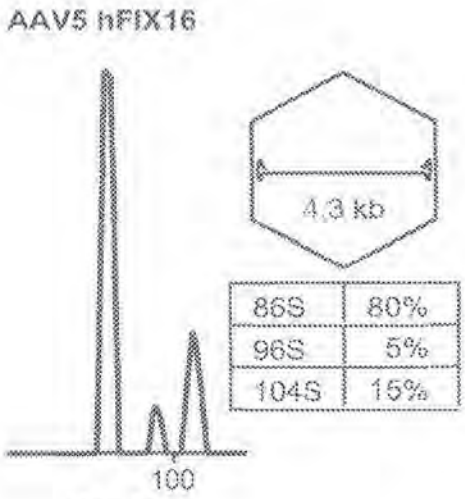


FIG. 11B

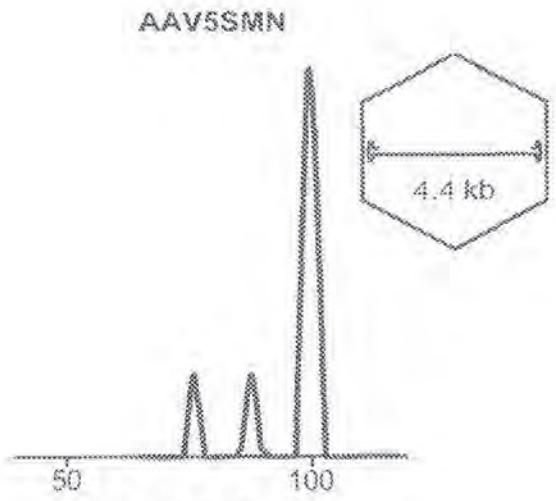


FIG. 11C

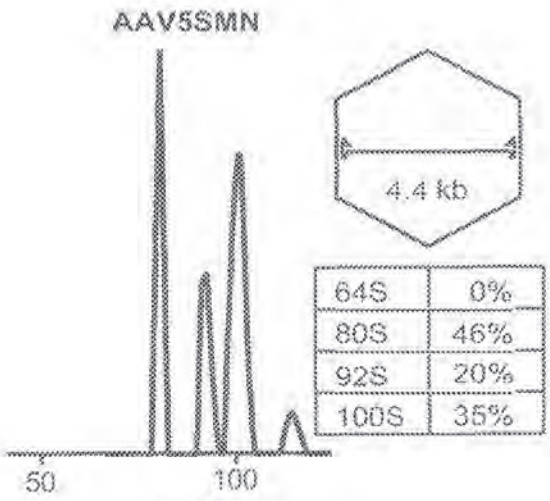


FIG. 11D

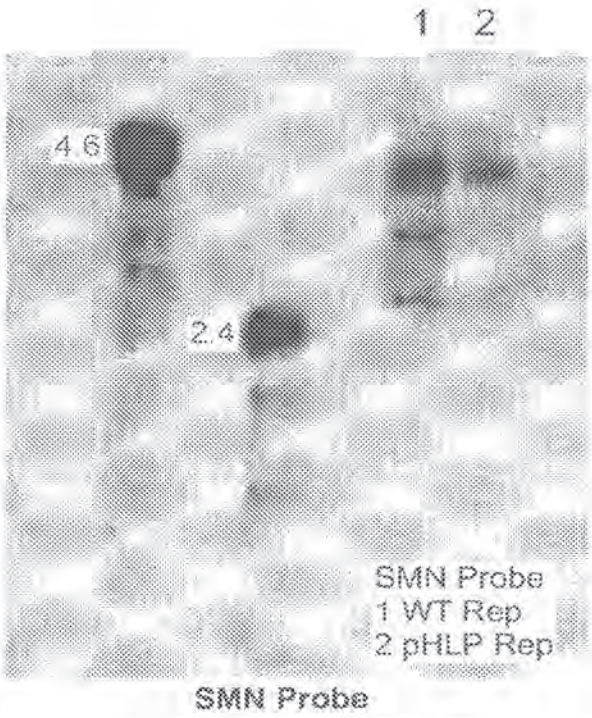


FIG. 12A

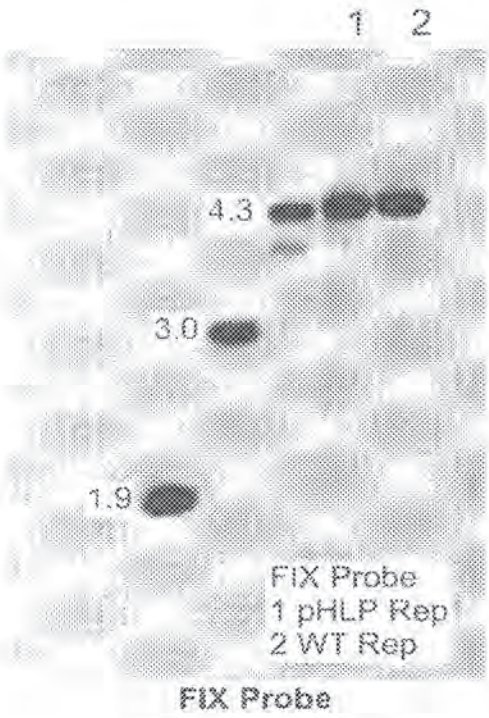


FIG. 12B

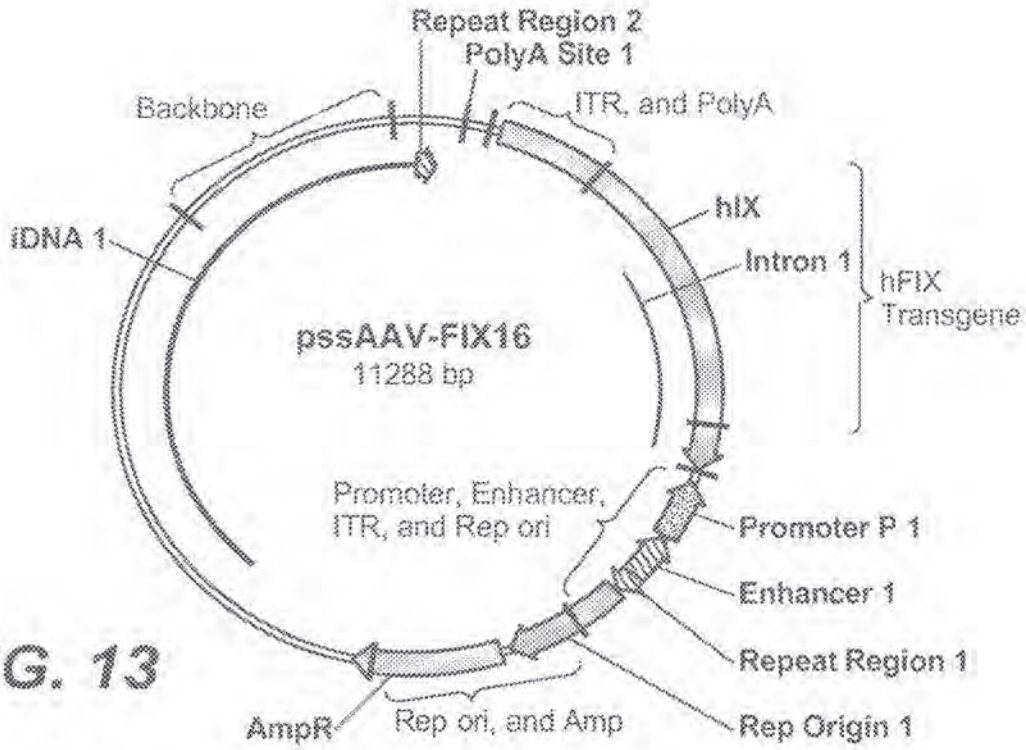
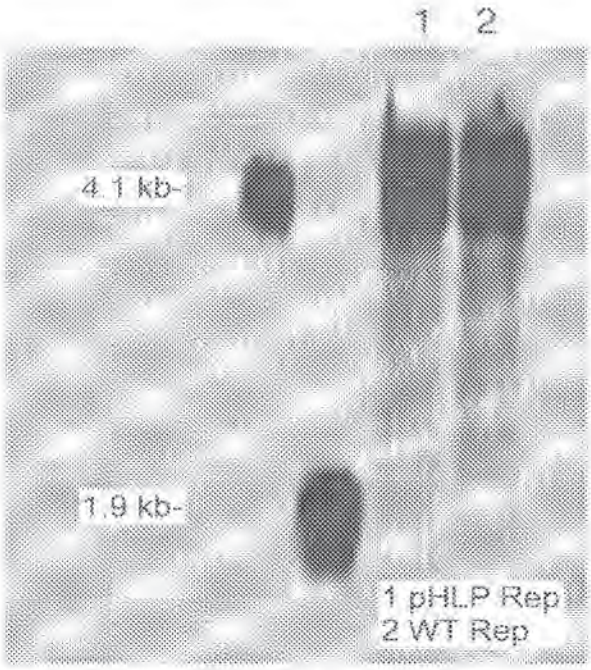
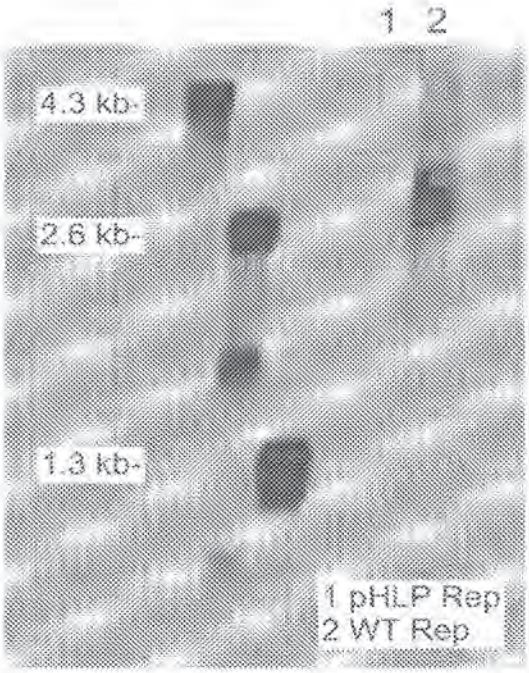


FIG. 13



Transgene

FIG. 14A



Rep ori & Amp

FIG. 14B

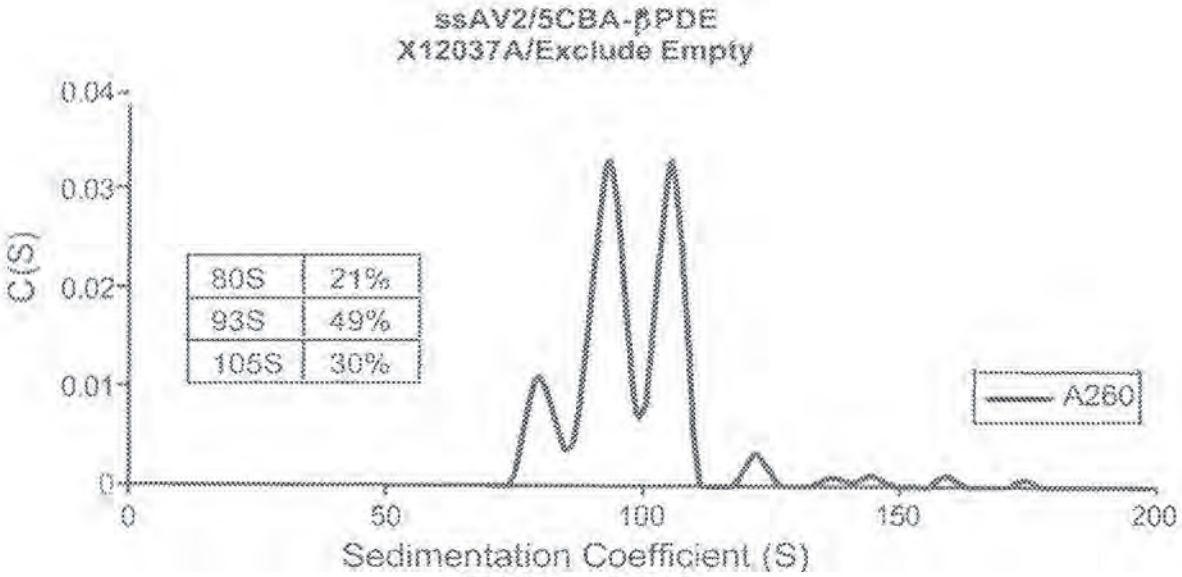


FIG. 15A

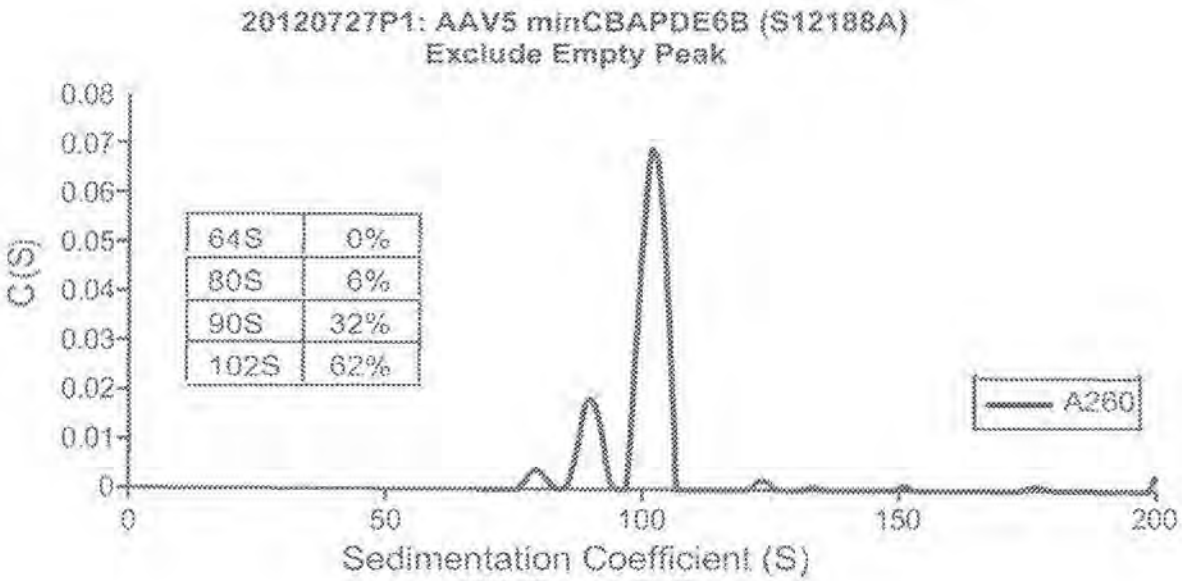


FIG. 15B

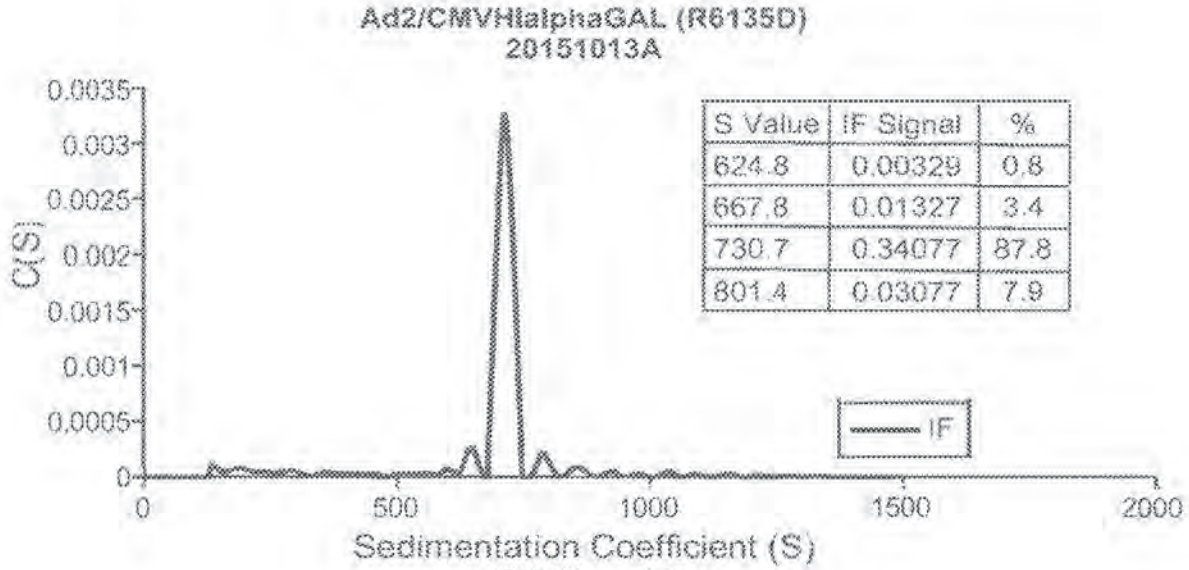

Ad2/CMVHIalphaGAL (R6135D)
20151013A
C(S)
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
500
1000
1500
2000
Sedimentation Coefficient (S)
IF
S Value | IF Signal | %
624.8 | 0.00329 | 0.8
667.8 | 0.01327 | 3.4
730.7 | 0.34077 | 87.8
801.4 | 0.03077 | 7.9

FIG. 16

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 18/188,176, filed Mar. 22, 2023, which is a continuation of U.S. patent application Ser. No. 16/547,144 (now U.S. Pat. No. 11,639,887), filed Aug. 21, 2019, which is a continuation of U.S. patent application Ser. No. 15/544,498 (now U.S. Pat. No. 10,429,288), which adopts the international filing date of Jan. 19, 2016, which is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2016/013947, filed Jan. 19, 2016, which claims priority to U.S. Provisional Application No. 62/105,714, filed Jan. 20, 2015, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

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

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).

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.

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

BRIEF SUMMARY OF THE INVENTION

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

In some embodiments of the above aspects, the following C(S) parameters are held constant: resolution of about 200 S to about 5000 S, S min is about 1 S to about 100 S, S max is about 100 S to about 5000 S, 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 200 S to about 1000 S. In some embodiments, resolution is about 200 S. In some embodiments, S min is about 1. In some embodiments, Smax is about 100 S to about 1000 S. In other embodiments, Smax is about 200 S to about 5000 S. In other embodiments, Smax is about 200 S. 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 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.

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.

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.

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.

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 **31**, 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 recombi-

nant 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.

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.

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.

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

FIGS. **1**A and **1**B show that analytical ultracentrifugation (AUC) can be used to characterize recombinant viral vector particles. (FIG. **1**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"). (FIG. **1**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 (%).

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

FIGS. **3**A and **3**B show a comparison between interference and absorbance detection methods by AUC. (FIG. **3**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. (FIG. **3**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.

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.

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.

FIGS. **6**A, **6**B and **6**C shows that vector production by the producer cell line and triple transfection methods yields different vector preparations, as revealed by AUC analysis. (FIG. **6**A) A schematic of the AAV2-transgene 2 vector and its 3.4 kb genome. (FIG. **6**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. (FIG. **6**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.

FIGS. **7**A, **7**B and **7**C show that the AUC method may be used to monitor the quality and efficacy of vector purification. (FIG. **7**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. (FIG. **7**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. (FIG. **7**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.

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.

FIGS. **9**A, **9**B and **9**C show that assessment of capsid genome size using AUC data correlates with assessment of genome size by Southern blot. (FIG. **9**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. (FIG. **9**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. (FIG. **9**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 (%).

FIGS. **10**A, **10**B and **10**C show that the Rep/Cap promoter position affects genome packaging in recombinant viral vectors produced by the triple transfection method. (FIG. **10**A) A schematic of the self-complementary scAAV2 EGFP vector, with estimated sedimentation coefficients for the dimeric and monomeric genome species. (FIG. **10**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 (80 S) and double stranded dimeric (100 S) species are labeled with the corresponding relative abundance values (%). (FIG. **10**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 (100 S) species are labeled with the corresponding relative abundance values (%).

FIGS. **11**A, **11**B, **11**C and **11**D show that the Rep/Cap promoter position affects genome packaging in two additional AAV vectors. (FIGS. **11**A and **11**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," FIG. **11**B) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," FIG. **11**A). (FIGS. **11**C-**11**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," FIG. **11**D) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," FIG. **11**C).

FIGS. **12**A and **12**B reveal that Southern blot analysis correlates with AUC analysis but misses some fragmented genomes detectable by AUC. (FIG. **12**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. (FIG. **12**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.

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.

FIGS. **14**A and **14**B 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. **14**A) Southern blot analysis using an hFIX transgene-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes. (FIG. **14**B) Southern blot analysis using a Rep ori/AmpR-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes.

FIGS. **15**A and **15**B show the fragmentation of oversized AAV vector genomes, as demonstrated by AUC analysis. (FIG. **15**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 (%). (FIG. **15**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 (%).

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

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.

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.

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.

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.

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.

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 recom-

binant viral preparation process) indicates an improvement in the production of recombinant viral particles.

I. General Techniques

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

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.

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.

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.

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).

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)".

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.

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."

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."

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."

"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.

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.

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.

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.

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).

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.

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.

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.

"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.

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.

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 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).

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.

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.

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.

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.

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.

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.

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.

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).

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.

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."

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.

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

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, S A & Philo J S, (2007) *Anal. Biochem.*, 362:16-37.

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.

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.

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.

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.

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.

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:

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

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.

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.

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.

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 1×10^7 vg/mL, 2×10^7 vg/mL, 3×10^7 vg/mL, 4×10^7 vg/mL, 5×10^7 vg/mL, 6×10^7 vg/mL, 7×10^7 vg/mL, 8×10^7 vg/mL, 9×10^7 vg/mL, 1×10^8 vg/mL, 2×10^8 vg/mL, 3×10^8 vg/mL, 4×10^8 vg/mL, 5×10^8 vg/mL, 6×10^8 vg/mL, 7×10^8 vg/mL, 8×10^8 vg/mL, 9×10^8 vg/mL, 1×10^9 vg/mL, 2×10^9 vg/mL, 3×10^9 vg/mL, 4×10^9 vg/mL, 5×10^9 vg/mL, 6×10^9 vg/mL, 7×10^9 vg/mL, 8×10^9 vg/mL, 9×10^9 vg/mL, 1×10^{10} vg/mL, 2×10^{10} vg/mL, 3×10^{10} vg/mL, 4×10^{10} vg/mL, 5×10^{10} vg/mL, 6×10^{10} vg/mL, 7×10^{10} vg/mL, 8×10^{10} vg/mL, 9×10^{10} vg/mL, 1×10^{11} vg/mL, 2×10^{11} vg/mL, 3×10^{11} vg/mL, 4×10^{11} vg/mL, 5×10^{11} vg/mL, 6×10^{11} vg/mL, 7×10^{11} vg/mL, 8×10^{11} vg/mL, 9×10^{11} vg/mL, 1×10^{12} vg/mL, 2×10^{12} vg/mL, 3×10^{12} vg/mL, 4×10^{12} vg/mL, 5×10^{12} vg/mL, 6×10^{12} vg/mL, 7×10^{12} vg/mL, 8×10^{12} vg/mL, 9×10^{12} vg/mL, 1×10^{13} vg/mL, 2×10^{13} vg/mL, 3×10^{13} vg/mL, 4×10^{13} vg/mL, 5×10^{13} vg/mL, 6×10^{13} vg/mL, 7×10^{13} vg/mL, 8×10^{13} vg/mL, 9×10^{13} vg/mL. In some embodiments, the viral particles are concentrated to of about 1×10^7 vg/mL to about 1×10^{13} vg/mL, about 1×10^8 vg/mL to about 1×10^{13} vg/mL, about 1×10^9 vg/mL to about 1×10^{13} vg/mL, about 1×10^{10} vg/mL to about 1×10^{13} vg/mL, about 1×10^{11} vg/mL to about 1×10^{13} vg/mL, about 1×10^{12} vg/mL to about 1×10^{13} vg/mL, about 1×10^7 vg/mL to about 1×10^{12} vg/mL, about 1×10^8 vg/mL to about 1×10^{12} vg/mL, about 1×10^9 vg/mL to about 1×10^{12} vg/mL, about 1×10^{10} vg/mL to about 1×10^{12} vg/mL, about 1×10^{11} vg/mL to about 1×10^{12} vg/mL, about 1×10^7 vg/mL to about 1×10^{11} vg/mL, about 1×10^8 vg/mL to about 1×10^{11} vg/mL, about 1×10^9 vg/mL to about 1×10^{11} vg/mL, about 1×10^{10} vg/mL to about 1×10^{11} vg/mL, about 1×10^7 vg/mL to about 1×10^{10} vg/mL, about 1×10^8 vg/mL to about 1×10^{10} vg/mL, about 1×10^9 vg/mL to about 1×10^{10} vg/mL, about 1×10^7 vg/mL to about 1×10^9 vg/mL, about 1×10^8 vg/mL to about 1×10^9 vg/mL, or about 1×10^7 vg/mL to about 1×10^8 vg/mL.

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 M M, et al., (2013) *Expert Opin Biol Ther.* 13(7):987-1011. Methods to purify HSV particles are found, for example, in Goins, W F et al., (2014) *Herpes Simplex Virus Methods in Molecular Biology* 1144:63-79.

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 260 nm 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.

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.

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 (2^{nd}) 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 200 S to about 5000 S, S min is about 1 S to about 100 S, S max is about 100 S to about 5000 S, 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 200 S, 300 S, 400 S, 500 S, 600 S, 700 S, 800 S, 900 S, or 1000 S. In some embodiments, the resolution is between any of about 200 S to about 1000 S, 200 S to about 900 S, 200 S to about 800 S, 200 S to about 700 S, 200 S to about 600 S, 200 S to about 500 S, 200 S to about 400 S, 200 S to about 300 S, 300 S to about 1000 S, 300 S to about 900 S, 300 S to about 800 S, 300 S to about 700 S, 300 S to about 600 S, 300 S to about 500 S, 300 S to about 400 S, 400 S to about 1000 S, 400 S to about 900 S, 400 S to about 800 S, 400 S to about 700 S, 400 S to about 600 S, 400 S to about 500 S, 500 S to about 1000 S, 500 S to about 900 S, 500 S to about 800 S, 500 S to about 700 S, 500 S to about 600 S, 600 S to about 1000 S, 600 S to about 900 S, 600 S to about 800 S, 600 S to about 700 S, 700 S to about 1000 S, 700 S to about 900 S, 700 S to about 800 S, 800 S to about 1000 S, 800 S to about 900 S, or 900 S to about 1000 S. In some embodiments, the resolution is about 200 S. In some embodiments, the Smax is about any of 100 S, 200 S, 300 S, 400 S, 500 S, 600 S, 700 S, 800 S, 900 S, or 1000 S. In some embodiments, the Smax is between any of about 100 S to about 1000 S, 100 S to about 900 S, 100 S to about 800 S, 100 S to about 700 S, 100 S to about 600 S, 100 S to about 500 S, 100 S to about 400 S, 100 S to about 300 S, 100 S to about 200 S, 200 S to about 1000 S, 200 S to about 900 S, 200 S to about 800 S, 200 S to about 700 S, 200 S to about 600 S, 200 S to about 500 S, 200 S to about 400 S, 200 S to about 300 S, 300 S to about 1000 S, 300 S to about 900 S, 300 S to about 800 S, 300 S to about 700 S, 300 S to about 600 S, 300 S to about 500 S, 300 S to about 400 S, 400 S to about 1000 S, 400 S to about 900 S, 400 S to about 800 S, 400 S to about 700 S, 400 S to about 600 S, 400 S to about 500 S, 500 S to about 1000 S, 500 S to about 900 S, 500 S to about 800 S, 500 S to about 700 S, 500 S to about 600 S, 600 S to about 1000 S, 600 S to about 900 S, 600 S to about 800 S, 600 S to about 700 S, 700 S to about 1000 S, 700 S to about 900 S, 700 S to about 800 S, 800 S to about 1000 S, 800 S to about 900 S, or 900 S to about 1000 S. In some embodiments, Smax is about 200 S to about 5000 S. In some embodiments, wherein Smax is about 200 S. 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.

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.

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.

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.

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.

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.

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 1 S and an S_{max} of about 1000 S. In some embodiments, data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with a resolution of about 200 S to about 1,000 S. In some embodiments, the resolution is about any of 200 S, 300 S, 400 S, 500 S, 600 S, 700 S, 800 S, 900 S, or 1000 S. In some embodiments, the resolution is between any of about 200 S to about 1000 S, 200 S to about 900 S, 200 S to about 800 S, 200 S to about 700 S, 200 S to about 600 S, 200 S to about 500 S, 200 S to about 400 S, 200 S to about 300 S, 300 S to about 1000 S, 300 S to about 900 S, 300 S to about 800 S, 300 S to about 700 S, 300 S to about 600 S, 300 S to about 500 S, 300 S to about 400 S, 400 S to about 1000 S, 400 S to about 900 S, 400 S to about 800 S, 400 S to about 700 S, 400 S to about 600 S, 400 S to about 500 S, 500 S to about 1000 S, 500 S to about 900 S, 500 S to about 800 S, 500 S to about 700 S, 500 S to about 600 S, 600 S to about 1000 S, 600 S to about 900 S, 600 S to about 800 S, 600 S to about 700 S, 700 S to about 1000 S, 700 S to about 900 S, 700 S to about 800 S, 800 S to about 1000 S, 800 S to about 900 S, or 900 S to about 1000 S. In some embodiments, the resolution is about 200 S. data analysis for rAAV, rHSV, lentiviral, and/or rAd particles is performed with an Smax of about any of 100 S, 200 S, 300 S, 400 S, 500 S, 600 S, 700 S, 800 S, 900 S, or 1000 S. In some embodiments, the Smax is between any of about 100 S to about 1000 S, 100 S to about 900 S, 100 S to about 800 S, 100 S to about 700 S, 100 S to about 600 S, 100 S to about 500 S, 100 S to about 400 S, 100 S to about 300 S, 100 S to about 200 S, 200 S to about 1000 S, 200 S to about 900 S, 200 S to about 800 S, 200 S to about 700 S, 200 S to about

600 S, 200 S to about 500 S, 200 S to about 400 S, 200 S to about 300 S, 300 S to about 1000 S, 300 S to about 900 S, 300 S to about 800 S, 300 S to about 700 S, 300 S to about 600 S, 300 S to about 500 S, 300 S to about 400 S, 400 S to about 1000 S, 400 S to about 900 S, 400 S to about 800 S, 400 S to about 700 S, 400 S to about 600 S, 400 S to about 500 S, 500 S to about 1000 S, 500 S to about 900 S, 500 S to about 800 S, 500 S to about 700 S, 500 S to about 600 S, 600 S to about 1000 S, 600 S to about 900 S, 600 S to about 800 S, 600 S to about 700 S, 700 S to about 1000 S, 700 S to about 900 S, 700 S to about 800 S, 800 S to about 1000 S, 800 S to about 900 S, or 900 S to about 1000 S. In some embodiments, Smax is about 200 S to about 5000 S. In some embodiments, wherein Smax is about 200 S. 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.

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 100 S to about 200 S, S min is about 1, S max is about 200 S to 300 S, and frictional ratio is about 1.0 to 1.2 S. In some embodiments, radial invariant (RI) and time invariant (TI) noise subtractions are applied.

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.

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.

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	260 nm 280 nm 230 nm	260 nm 280 nm 230 nm	260 nm 280 nm 230 nm	260 nm 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

TABLE 1-continued

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

	AAV	Ad	Lentivirus	HSV
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.

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.

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.

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.

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.

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.

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.

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.

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

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).

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. Viral.* 2004, 78(12):6381).

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.

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

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 U.S. Pat. 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 non-coding 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.

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 (~950 Å), non-enveloped icosahedral capsid. Adenoviruses have a large genome that can incorporate more than 30 kb 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.

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, AdHu3, 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.

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. Viral.* 72:8463-71). For further description, see Durand, S. and Cimarelli, A. (2011) *Viruses* 3:132-59.

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 (Hy), 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.

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.

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

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, J E 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. Pat. No. 6,566,118, and Sf-900 II SFM media as described in U.S. Pat. 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).

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.

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 U.S. Pat. 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.

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.

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. Pat. 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.

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.

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 M M, et al., (2013) *Expert Opin Biol Ther.* 13(7):987-1011) described herein.

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, W F et al., (2014) *Herpes Simplex Virus Methods in Molecular Biology* 1144:63-79).

VI. Purification of rAAV Vectors

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.

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.

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.

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; U.S. Pat. Nos. 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 M M, et al., (2013) *Expert Opin Biol Ther.* 13(7):987-1011.

Methods to purify HSV particles are found, for example, in Goins, W F et al., (2014) *Herpes Simplex Virus Methods in Molecular Biology* 1144:63-79.

EXAMPLES

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

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, M W & Carter, B J, 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.

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

In order to support accurate AUC assessment, vector product (AAV2-transgene 2) was highly purified, suitably buffered, and concentrated to greater than 5×10^{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 260 nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260 nm 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 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

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 (2^{nd}) 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=200 S, 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)

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

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

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

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

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

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 260 nm along a centrifugal field at defined time intervals. FIG. **1**A 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. **1**A).

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. **1**B). The C(S) values were determined using the SEDFIT algorithm described by Schuck (2000) *Biophys.* 1, 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 ($Abs_{260\ nm}$)	$\varepsilon_{260\ nm}$	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	

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.

For the rAAV2 vector prep shown in FIG. **1**, capsids containing a full genome were represented by the peak sedimenting at 94 S and accounted for 21% of the vector prep. Empty capsids sedimented with an S value of 64 S and accounted for 79% of the vector prep. These sedimentation coefficient values were confirmed by AUC analysis of pure populations of empty (FIG. **2**A) or genome containing particles (FIG. **2**B). The AUC profile of a pure population of rAAV2 empty capsids revealed a single peak with a sedimentation coefficient of 64 S, whereas the AUC profile of a pure population of rAAV2AUC genome-containing capsids revealed a single peak with a higher sedimentation coefficient of 94 S. 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.

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.

		Full Capsid, Peak 3	
AUC Run	Empty Capsid, Peak 1 (S)	% 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

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.

Pure populations of empty and genome-containing AAV2 capsids were mixed at a 1:1 ratio and analyzed by AUC using both interference (FIG. **3**A) and absorbance detection methods (FIG. **3**B). Interference detection revealed two populations of AAV capsids at the approximate expected ratios of 43% empty and 57% genome-containing (FIG. **3**A). 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 FIGS. **3**A and **3**B). 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.

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

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

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.

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. **6**A shows a schematic of this AAV2 vector genome.

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 92 S species (FIG. **6**B). 19% were empty capsids, with the remainder containing a fragmented genome (75 S species, 7%). In contrast, 82% of the capsids produced by the triple transfection method were empty, 64 S species (FIG. **6**C), with 11% of capsids containing a fragmented genome (76 S and 84 S species) and only 8% of capsids having a full genome (94S).

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

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. **7**A). 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. **7**A).

As shown in FIG. **7**B, 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 92 S. In contrast, the rAAV2 vector prep prior to the chromatographic step (FIG. **7**C) had a substantial level of empty capsids. AUC analysis revealed two peaks with S values of 63 and 93, with the 63 S 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

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.

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) (y)	Genome size (# NT) (x)	MW	Extinction coefficient (260 nm)
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

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 (AAV2/9minCBAE-GFP; see schematic in FIG. **9**A). 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 101 S species, representing an encapsidated genome of ~4.3 kb (FIG. **9**A). This 101 S peak represented the double stranded dimeric vector genome, which has a predicted size of ~4.3 kb. However, the majority of the vector prep (75%), sedimented with an S value of 82, which corresponds to a vector genome size of ~2 kb (FIG. **9**A), 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).

FIG. **9**B shows an alkaline Southern blot of the same vector, scAAV9 EGFP, which revealed two vector populations with genomes 4.3 kb and ~2 kb in size, corroborating the AUC data in FIG. **9**A. 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 (~4 kb) revealed a single predominant peak with a measured S value of 99 S, corresponding to approximately 4.1 kb by the standard curve and 84% of capsid abundance (FIG. **9**C). 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 ~4 kb (lane 2, FIG. **9**B). 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

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

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. **10**A) was produced using one of two methods. In the first method (FIG. **10**B), 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. **10**C), 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 100 S in dimeric genome form and 80 S in monomeric genome form (FIG. **10**A).

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 100 S species (FIG. **10**C). This was the expected sedimentation coefficient for a capsid containing a dimeric genome of 4.4 kb. 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 80 S (FIG. **10**B). 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.

A single stranded AAV5 Factor IX vector, AAV5FIX, (FIGS. **11**A-B) and a single stranded AAV5hSMN vector (FIG. **11**C-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 (4.3 kb), the predicted sedimentation coefficient for the AAV5 FIX vector capsid was approximately 101 S. 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, ~101 S (FIG. **11**A). 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. **11**B). Instead of a predominant peak at 101 S, this profile revealed more capsid heterogeneity, with the majority of the AAV5 FIX (80%) sedimenting at a lower S value of 86 S, 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 ~104 S (FIG. **11**B).

AAV5 SMN 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 101 S, consistent with packaging of a genome of the predicted size of ~4.4 kb (FIG. **11**C). 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 100 S (predicted S value for the full vector genome of 4400 nt), 92 S (representing a fragmented genome of approximately 3300 nt) and 80 S (representing a fragmented genome of 2000 nucleotides) (FIG. **11**D). These results confirm a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid using two additional AAV vectors.

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 (4.4 kb) and fragmented (less than 4.4 kb) SMN genomes (FIG. **12**A, 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. **12**A, 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. **12**B, lane 2). However, AUC analysis of this AAV5FIX vector (FIG. **11**B) showed that 80% of the capsids contained a fragmented genome (~3000 nucleotides), which were undetected by the FIX probe.

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. **14**A, both vector preparations (pHLP rep, lane 1; WT rep, lane 2) contained the hFIX transgene. However, FIG. **14**B lane 2 confirms that the vector genome species sedimenting at 86 S observed in the WT Rep preparation (FIG. **11**B) was a ~3 kb fragment. Moreover, this species reacted with an Amp^R specific probe (FIG. **14**B, 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. **14**B, lane 1).

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.

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 5.4 kb (FIG. **15**A) or as a wild type size genome of 4.6 kb (FIG. **15**B). To generate the 4.6 kb genome, the

CBA promoter was truncated by reducing the size of the intron as previously reported (Gray, S J et al., (2011) *Hum. Gene Ther.* 22(9):1143-1153).

As shown in FIG. **15**A, 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 93 S species, consistent with packaging a fragmented vector genome of approximately 3.5 kb. 30% of the preparation was represented by another sub-genomic vector species of approximately 4.9 kb sedimenting at 105 S. There was no evidence of packaging of a full-length 5.4 kb genome, which was predicted to sediment at 108-109 S. In contrast, AUC analysis revealed that the same transgene under control of the abbreviated CBA promoter sedimented predominantly as a vector species of 102 S, consistent with packaging of the predicted, full-length vector genome of 4.6 kb (FIG. **15**B). 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.

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

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

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 260 nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260 nm 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

The percent full capsid was determined by analyzing approximately 75 scans from interference detection method using the SEDFIT (NIH/see worldwide web at analytical-ultracentrifugation.com) continuous size C(S) distribution model. Second (2^{nd}) 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=2505, 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

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.* 1, 78:1606-19.

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.

What is claimed is:

1. A method of quantifying one or more species of individual variant viral particles comprising fragmented recombinant adeno-associated viral (rAAV) genomes in a heterogeneous mixture of viral particles, said method comprising:

(i) subjecting the heterogeneous mixture of viral particles to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate sedimenting boundaries, wherein the boundary sedimentation velocity is from about 3,000 rpm to about 20,000 rpm;

(ii) measuring the rate of movement or migration of the sedimenting boundaries, wherein movement or migration of each species of individual viral particles in the heterogeneous mixture of viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable species of viral particle, and wherein the individual viral particles comprise empty particles without genome, particles with full genomes and particles with fragmented genomes;

(iii) determining the genome size of one or more species of the individual variant viral particles in the heterogeneous mixture of viral particles; and

(iv) determining the quantity of one or more species of the variant viral particles in the heterogeneous mixture of viral particles.

2. The method of claim 1, wherein each species of the individual variant viral particles in a heterogeneous mixture of viral particles is fully resolved.

3. The method of claim 1, wherein determining the genome size of one or more species of the individual variant viral particles in the heterogeneous mixture of viral particles comprises comparing the sedimentation coefficients of the variant viral particles with a standard curve generated by sedimentation coefficients of viral particles comprising recombinant AAV genomes of known nucleotide sizes.

4. The method of claim 1, wherein the boundary sedimentation velocity is from about 10,000 rpm to about 20,000 rpm.

5. The method of claim 3, wherein the boundary sedimentation velocity is from about 10,000 rpm to about 20,000 rpm.

6. The method of claim 1, wherein the boundary sedimentation velocity is from about 15,000 rpm to about 20,000 rpm.

7. The method of claim 3, wherein the boundary sedimentation velocity is from about 15,000 rpm to about 20,000 rpm.

8. The method of claim 1, wherein the boundary sedimentation is performed at a temperature of about 4° C.

9. The method of claim 3, wherein the boundary sedimentation is performed at a temperature of about 4° C.

10. The method of claim 1, wherein the fragmented genomes are sub-genomic DNA molecules.

11. The method of claim 3, wherein the fragmented genomes are sub-genomic DNA molecules.

12. The method of claim 1, wherein the AUC is run from about 0.5 hours to about 2 hours.

13. The method of claim 3, wherein the AUC is run from about 0.5 hours to about 2 hours.

14. The method of claim 12, wherein the AUC is run from about 1 hours to about 2 hours.

15. The method of claim 13, wherein the AUC is run from about 1 hours to about 2 hours.

16. The method of claim 1, wherein the AUC is run in an ultracentrifuge velocity cells with two sectors in dialysis equilibrium, wherein each sector comprises an optical window.

17. The method of claim 3, wherein the AUC is run in an ultracentrifuge velocity cells with two sectors in dialysis equilibrium, wherein each sector comprises an optical window.

18. The method of claim 16, wherein the AUC is run until the lightest sedimenting component clears the optical window.

19. The method of claim 17, wherein the AUC is run until the lightest sedimenting component clears the optical window.

20. The method of claim 1, wherein the total concentration of viral particles in the heterogeneous mixture of viral particles prior to step (i) is greater than 5×10^{11} vg/mL.

21. The method of claim 3, wherein the total concentration of viral particles in the heterogeneous mixture of viral particles prior to step (i) is greater than 5×10^{11} vg/mL.

22. The method of claim 1, wherein the total concentration of viral particles in the heterogeneous mixture of viral particles AAV vector preparation prior to step (i) is from about 1×10^{11} vg/mL to about 1×10^{13} vg/mL.

23. The method of claim 3, wherein the total concentration of viral particles in the heterogeneous mixture of viral particles AAV vector preparation prior to step (i) is from about 1×10^{11} vg/mL to about 1×10^{13} vg/mL.

24. The method of claim 1, wherein the heterogeneous mixture of viral particles comprises viral particles comprising AAV9 capsid proteins.

25. The method of claim 24, wherein the viral particles comprising AAV9 capsid proteins further comprise at least one AAV2 ITR.

26. The method of claim 24, wherein the heterogeneous mixture of viral particles preparation comprise viral particles encapsidating self-complementary AAV viral genomes.

27. The method of claim 3, wherein the heterogeneous mixture of viral particles comprises viral particles comprising AAV9 capsid proteins.

28. The method of claim 27, wherein the viral particles comprising AAV9 capsid proteins further comprise at least one AAV2 ITR.

29. The method of claim 27, wherein the heterogeneous mixture of viral particles preparation comprise viral particles encapsidating self-complementary AAV viral genomes.

30. The method of claim 1, further comprising determining the relative percentage of each species of the individual viral particles in the heterogeneous mixture of viral particles.

* * * * *

Exhibit P

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(12) **United States Patent**
O'Riordan et al.

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(54) **ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES**

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(63) Continuation of application No. 18/188,176, filed on Mar. 22, 2023, which is a continuation of application No. 16/547,144, filed on Aug. 21, 2019, now Pat. No. 11,639,887, which is a continuation of application No. 15/544,498, filed as application No. PCT/US2016/013947 on Jan. 19, 2016, now Pat. No. 10,429,288.

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See application file for complete search history.

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(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 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.

30 Claims, 17 Drawing Sheets

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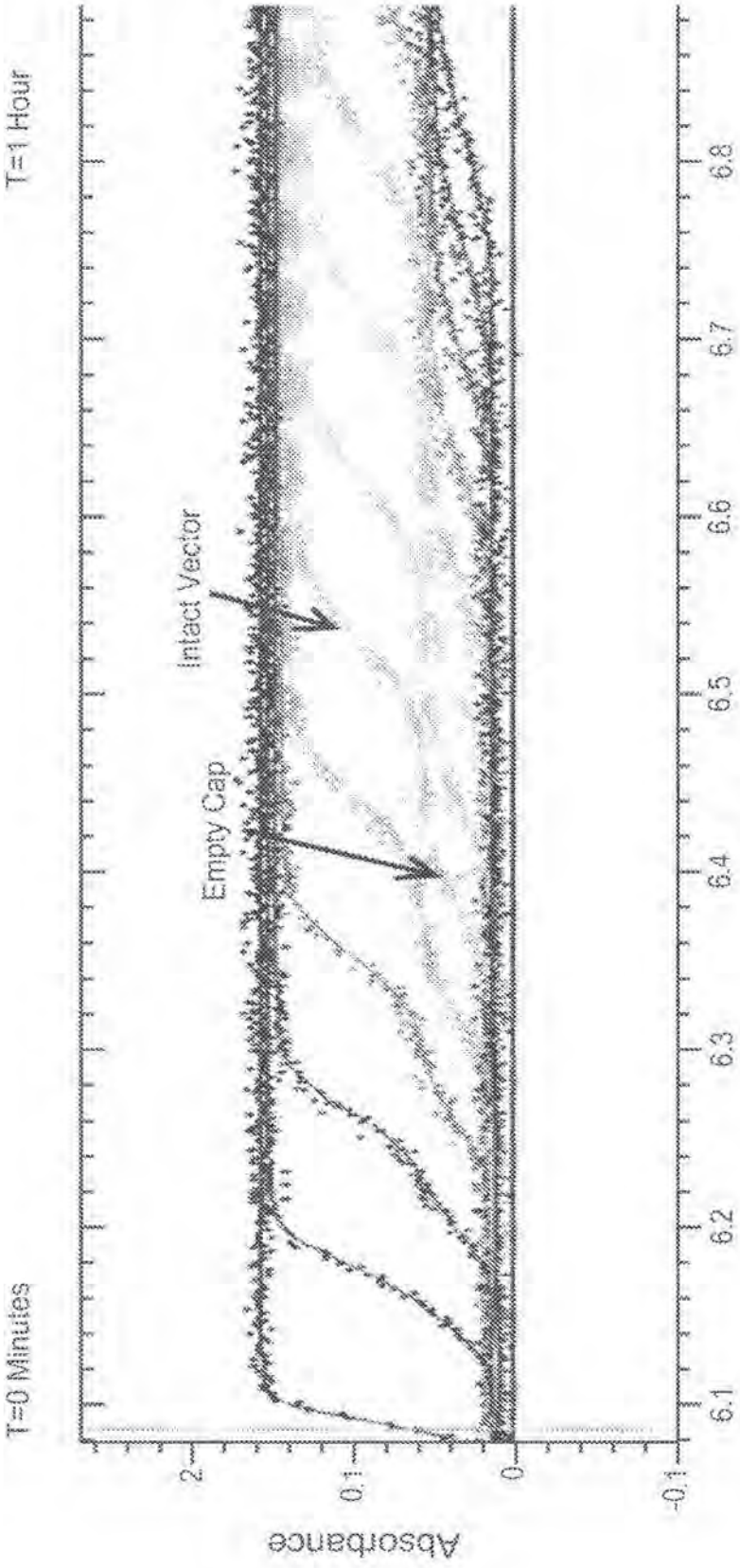

T=1 Hour
T=0 Minutes
Intact Vector
Empty Cap
Absorbance
2
0.1
0
-0.1
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
Radius, cm

FIG. 1A

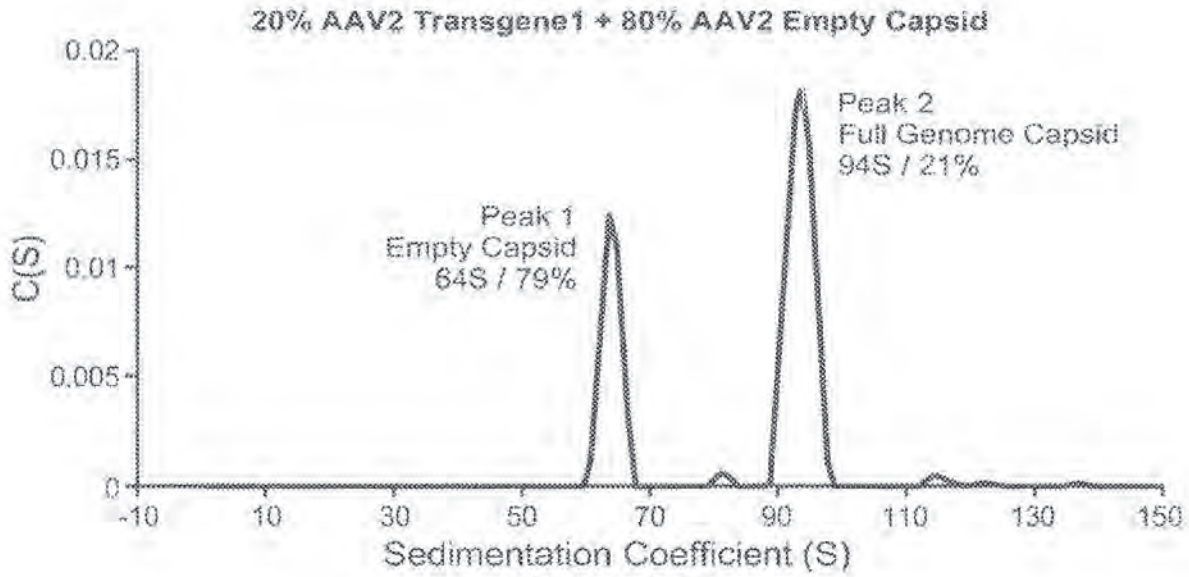

20% AAV2 Transgene1 + 80% AAV2 Empty Capsid
0.02
0.015
0.01
0.005
0
C(S)
Peak 1
Empty Capsid
64S / 79%
Peak 2
Full Genome Capsid
94S / 21%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 1B

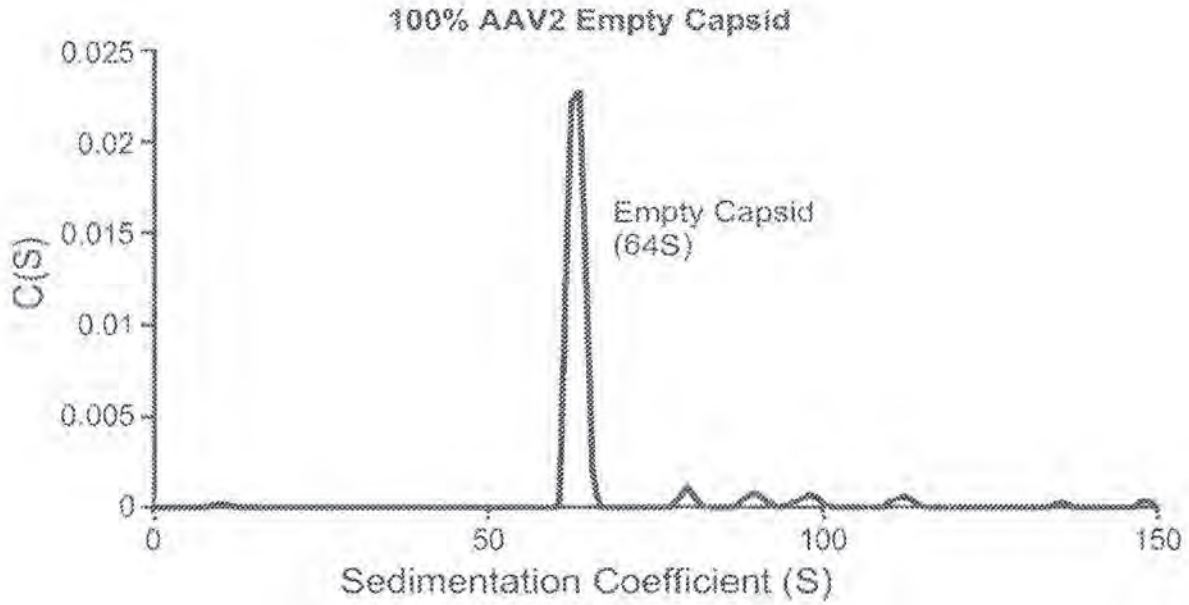

100% AAV2 Empty Capsid
0.025
0.02
0.015
0.01
0.005
0
C(S)
Empty Capsid
(64S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2A

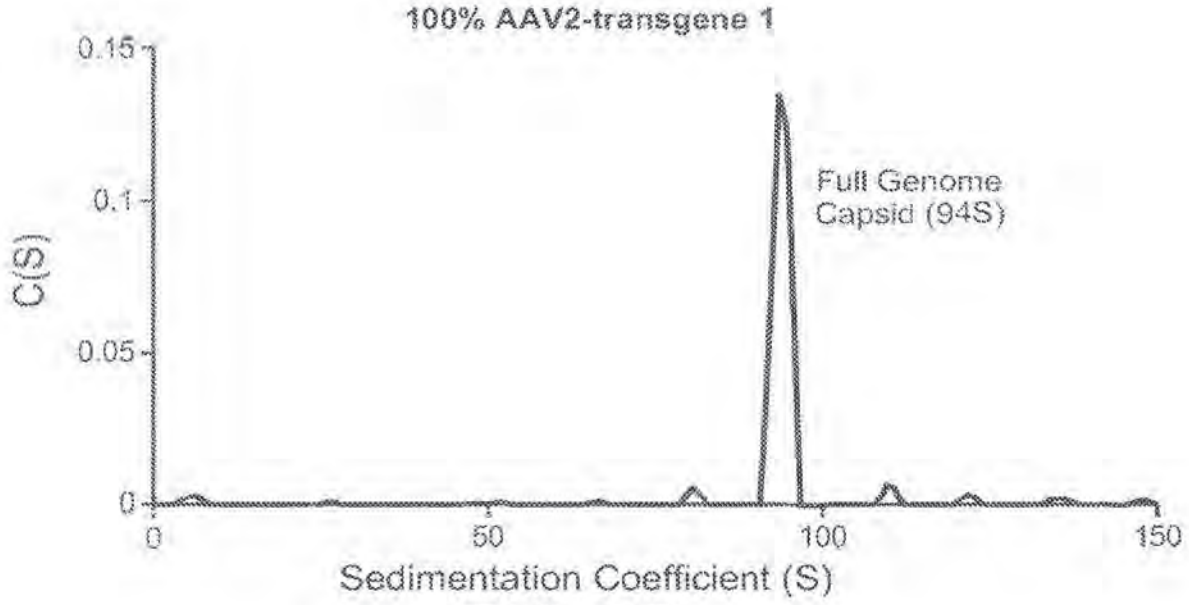

100% AAV2-transgene 1
0.15
0.1
0.05
0
C(S)
Full Genome
Capsid (94S)
0
50
100
150
Sedimentation Coefficient (S)

FIG. 2B

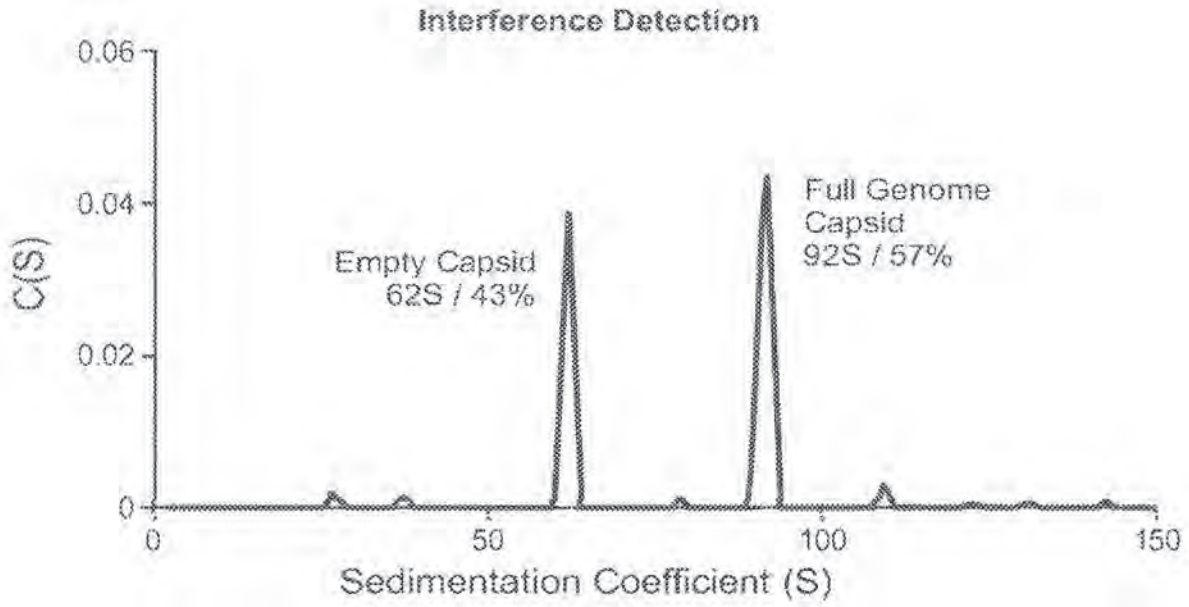

Interference Detection
0.06
0.04
0.02
0
C(S)
Empty Capsid
62S / 43%
Full Genome
Capsid
92S / 57%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3A

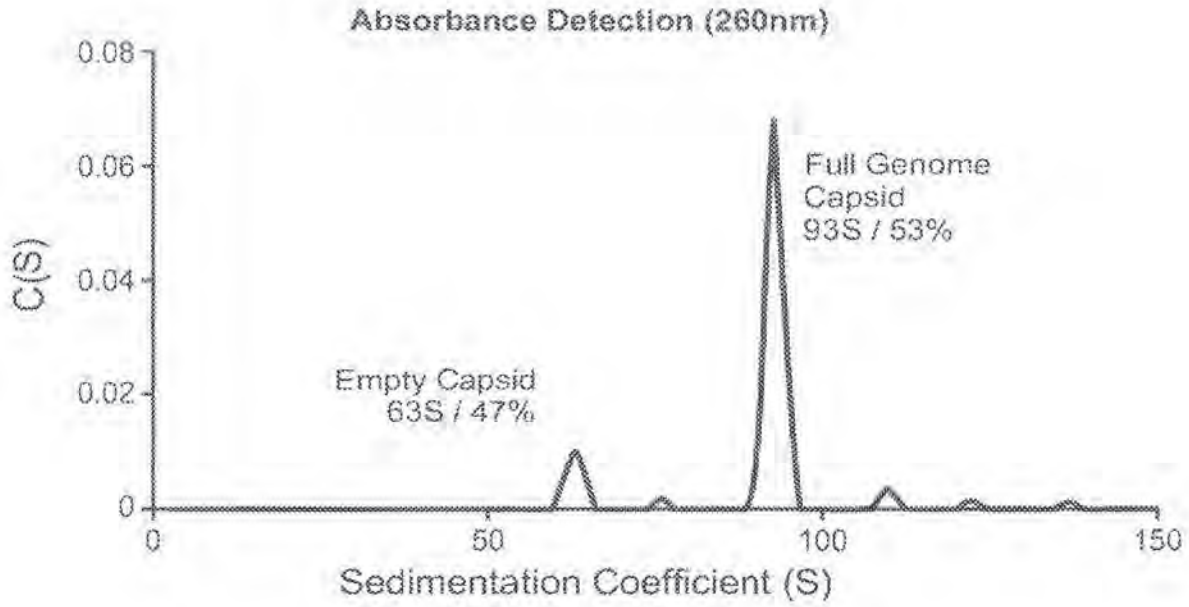

Absorbance Detection (260nm)
0.08
0.06
0.04
0.02
0
C(S)
Empty Capsid
63S / 47%
Full Genome
Capsid
93S / 53%
0
50
100
150
Sedimentation Coefficient (S)

FIG. 3B

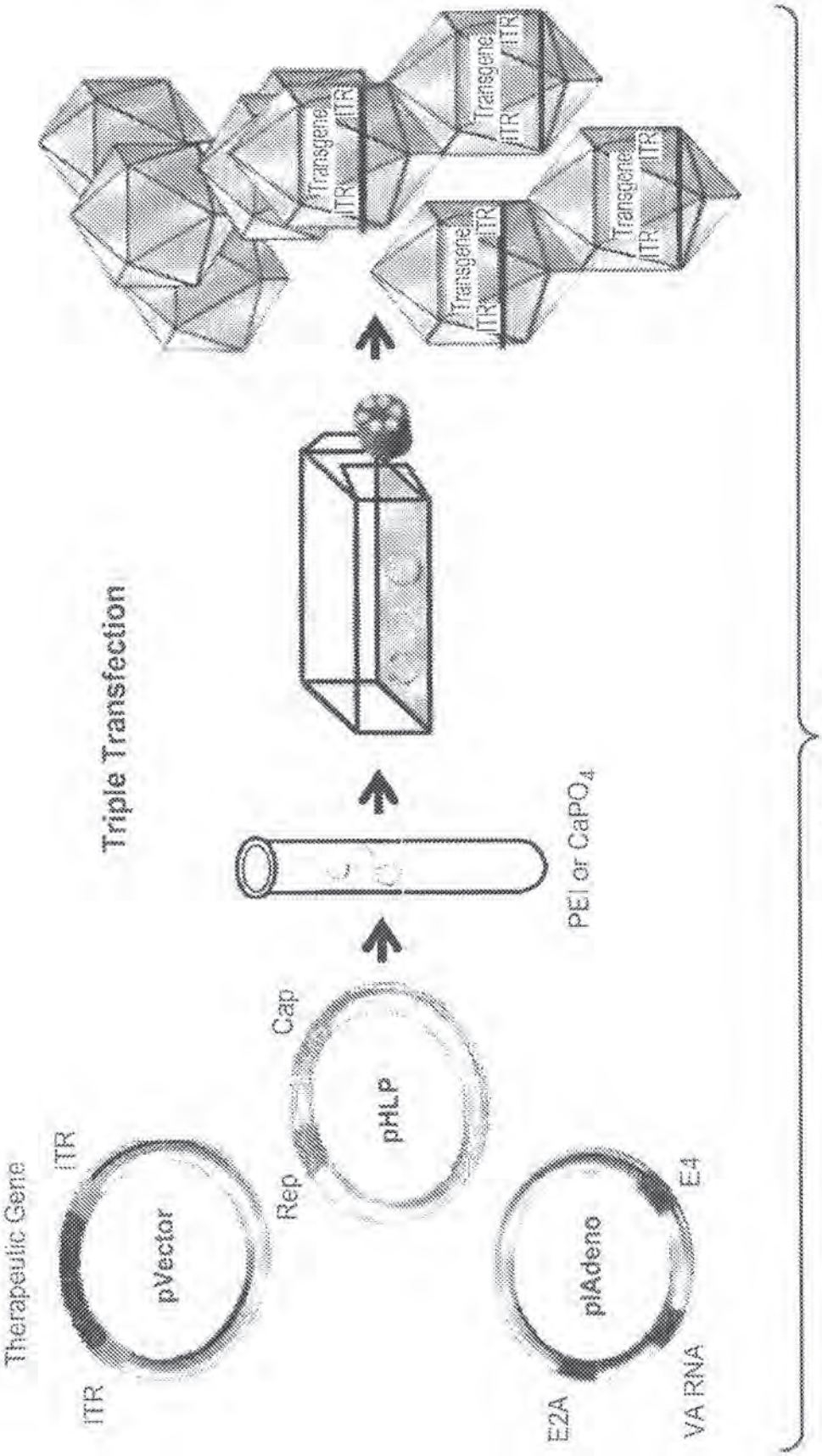

Therapeutic Gene
ITR
pVector
ITR
Rep
Cap
pHLP
E2A
E4
VA RNA
pAdeno
PEI or $CaPO_4$
Triple Transfection
Transgene
ITR

FIG. 4

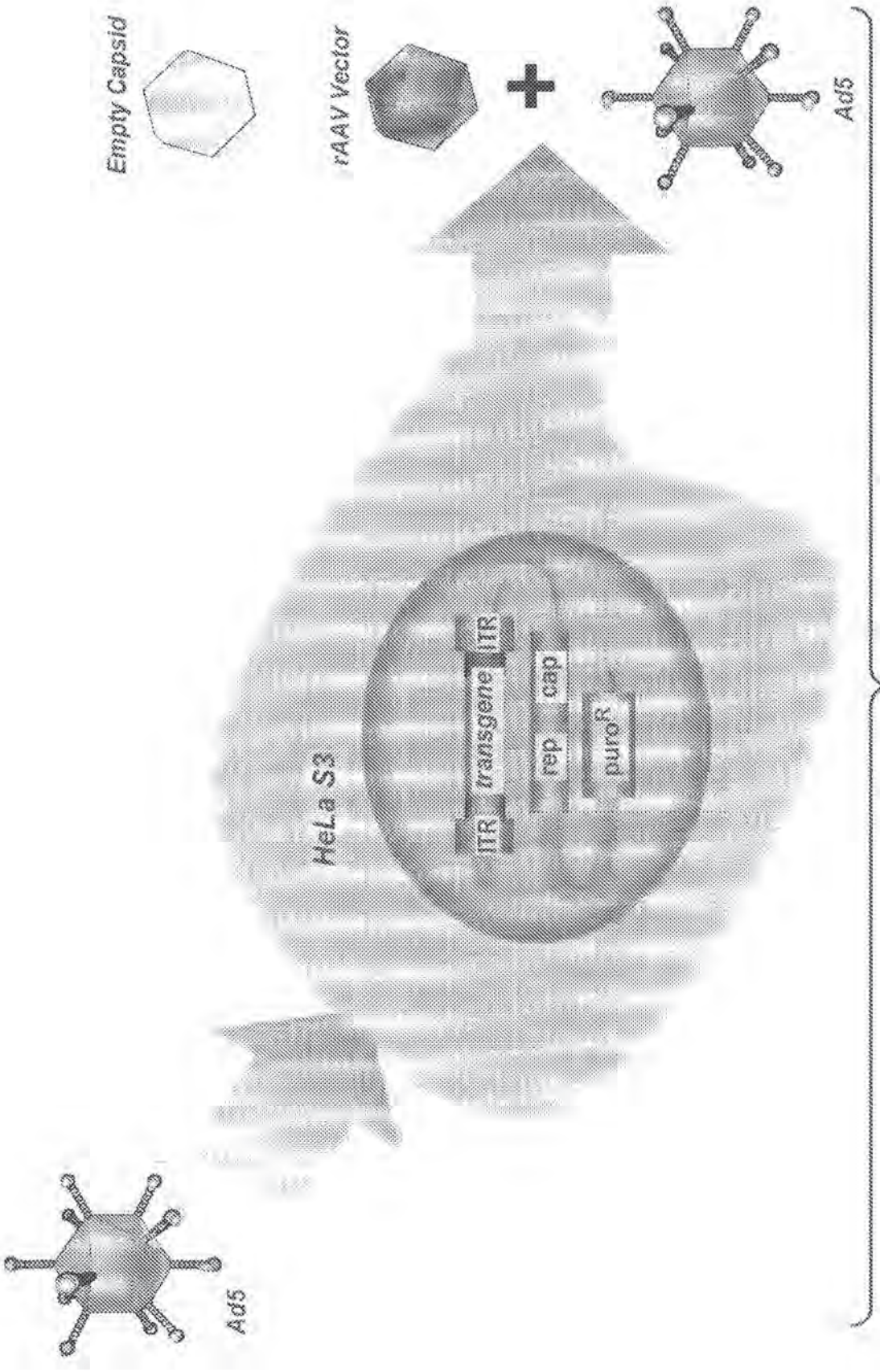
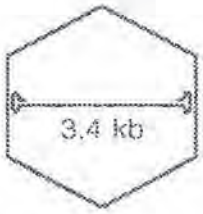

Empty Capsid
rAAV Vector
Ad5
HeLa S3
ITR
transgene
ITR
rep
cap
puroR
Ad5

FIG. 5

FIG. 6A



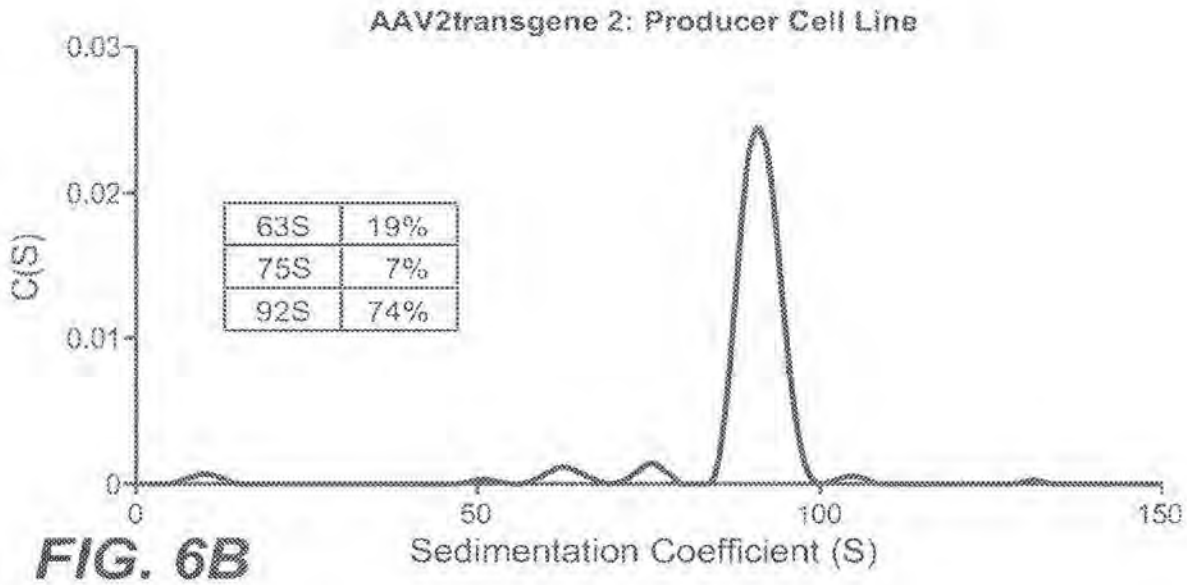


FIG. 6B

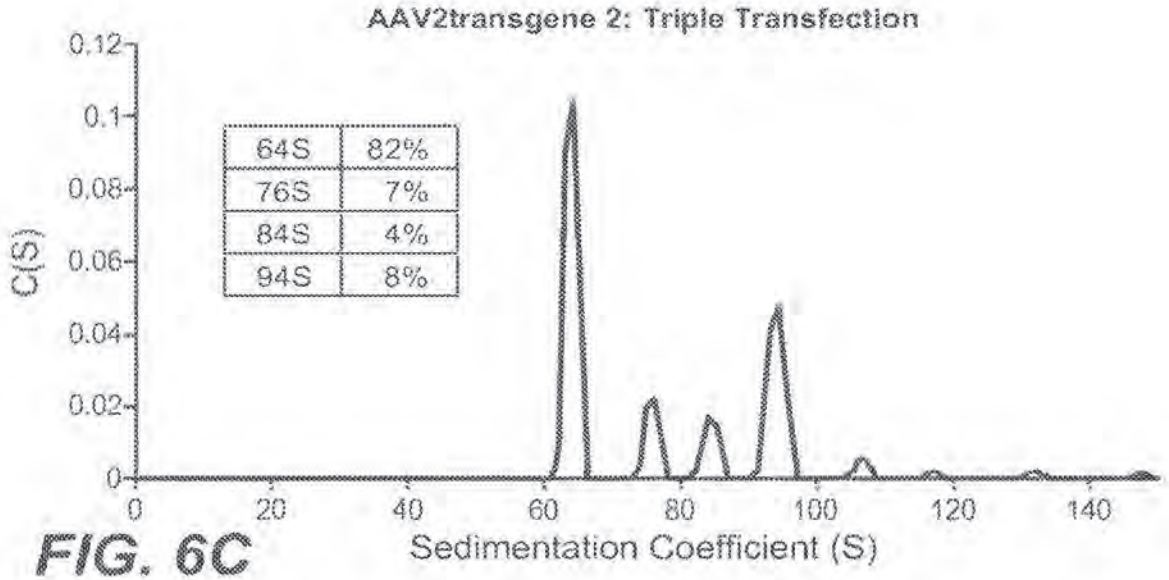


FIG. 6C

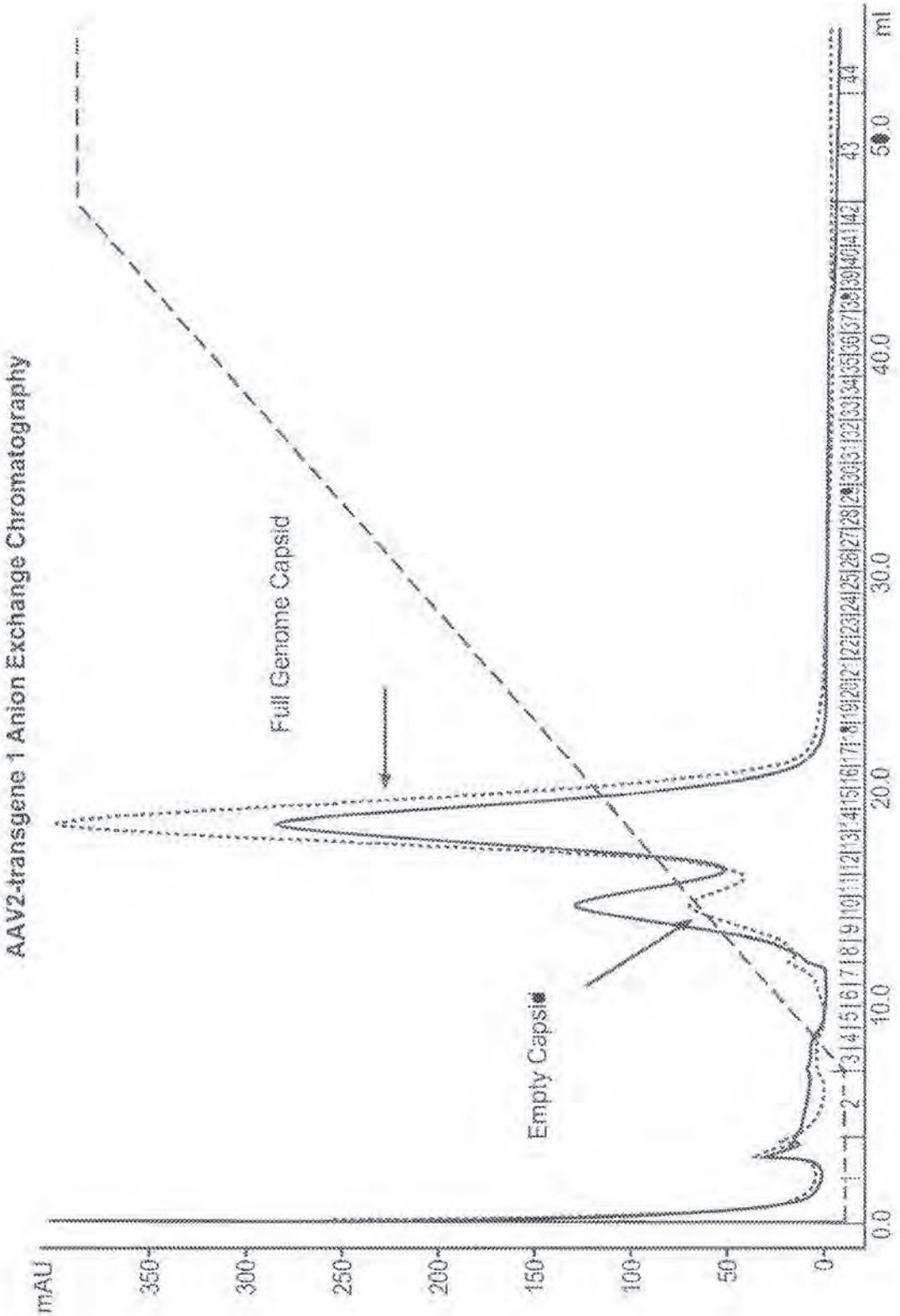

AAV2-transgene 1 Anion Exchange Chromatography
Full Genome Capsid
Empty Capsid
mAU
350
300
250
200
150
100
50
0
0.0
10.0
20.0
30.0
40.0
50.0
ml

FIG. 7A

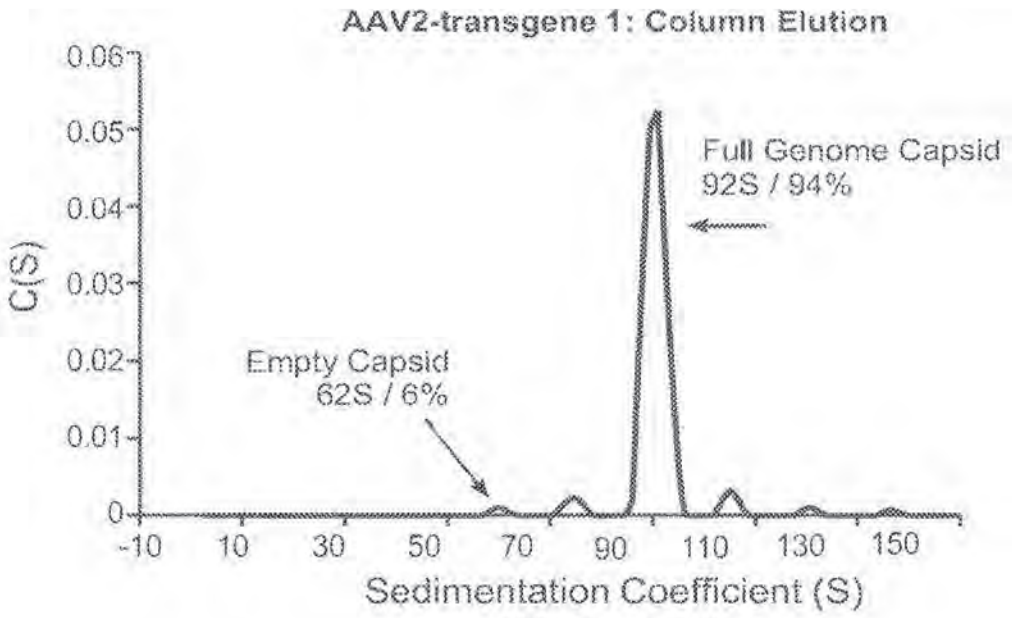

AAV2-transgene 1: Column Elution
0.06
0.05
0.04
0.03
0.02
0.01
0
C(S)
Full Genome Capsid
92S / 94%
Empty Capsid
62S / 6%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7B

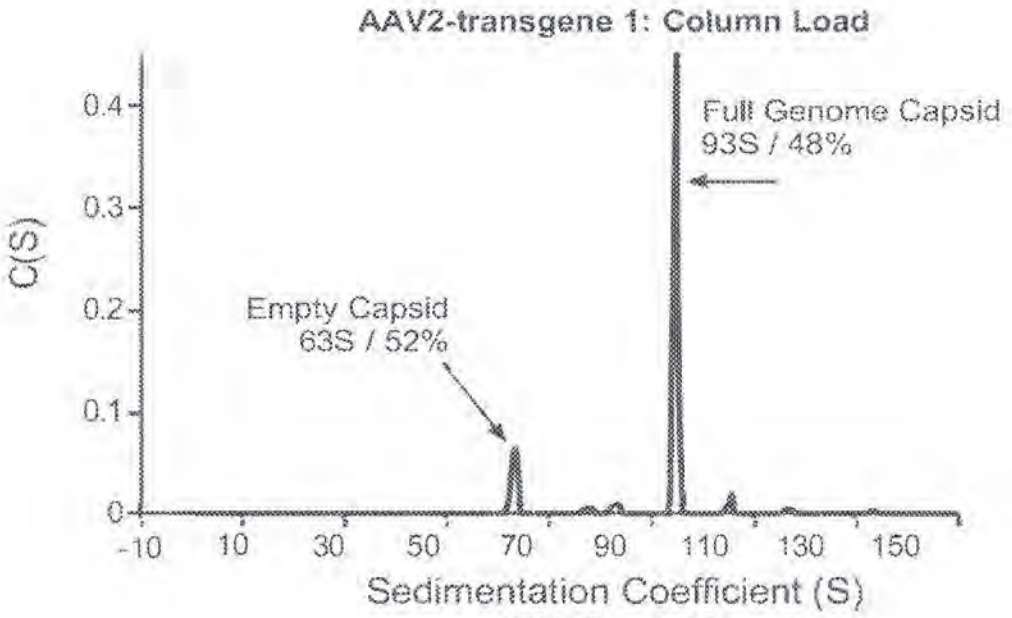

AAV2-transgene 1: Column Load
0.4
0.3
0.2
0.1
0
C(S)
Full Genome Capsid
93S / 48%
Empty Capsid
63S / 52%
-10
10
30
50
70
90
110
130
150
Sedimentation Coefficient (S)

FIG. 7C

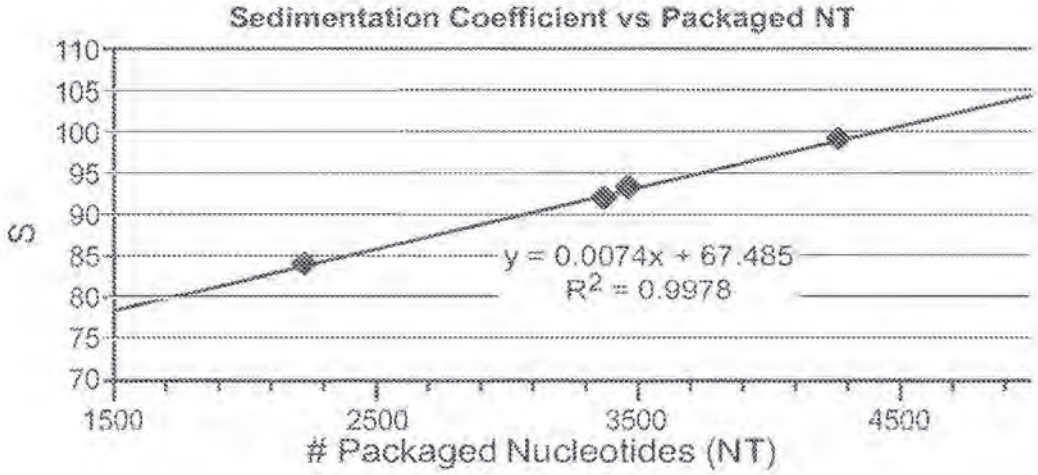

Sedimentation Coefficient vs Packaged NT
S
110
105
100
95
90
85
80
75
70
y = 0.0074x + 67.485
R2 = 0.9978
1500
2500
3500
4500
Packaged Nucleotides (NT)

FIG. 8

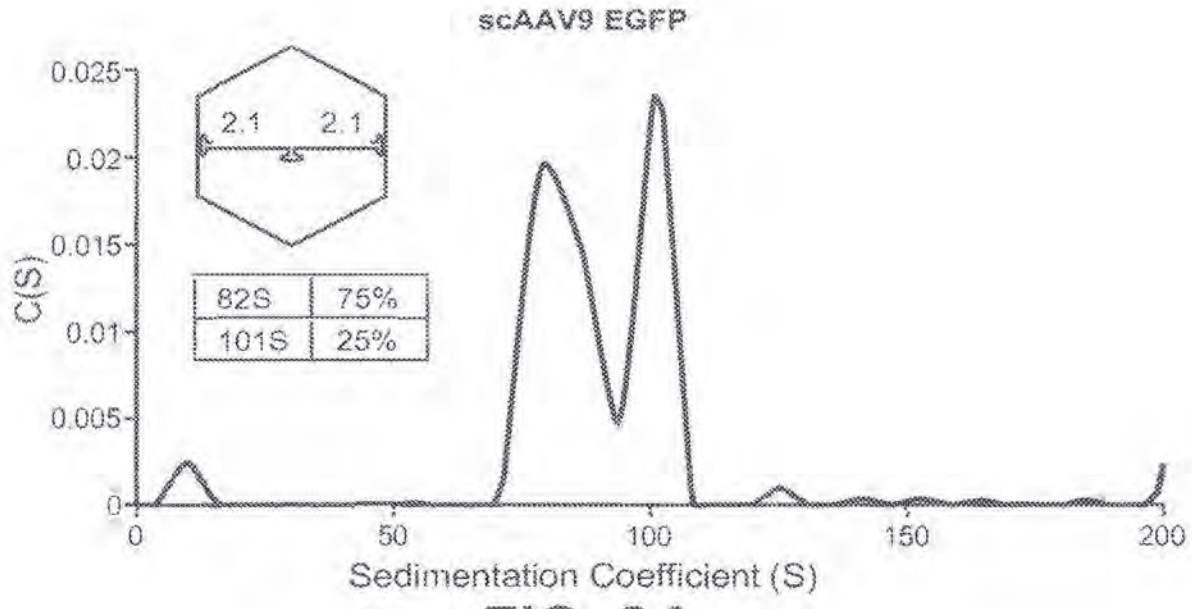

scAAV9 EGFP
C(S)
0.025
0.02
0.015
0.01
0.005
0
2.1
2.1
82S 75%
101S 25%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9A

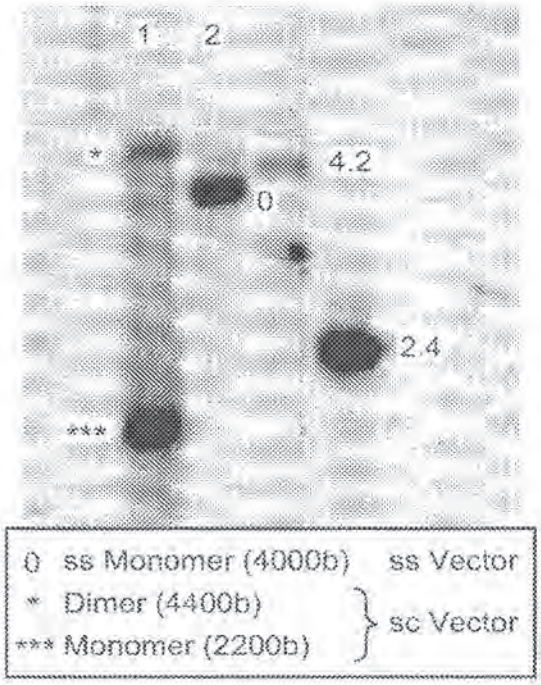

1
2
*
4.2
0
2.4
***
0 ss Monomer (4000b)
* Dimer (4400b)
*** Monomer (2200b)
ss Vector
sc Vector

FIG. 9B

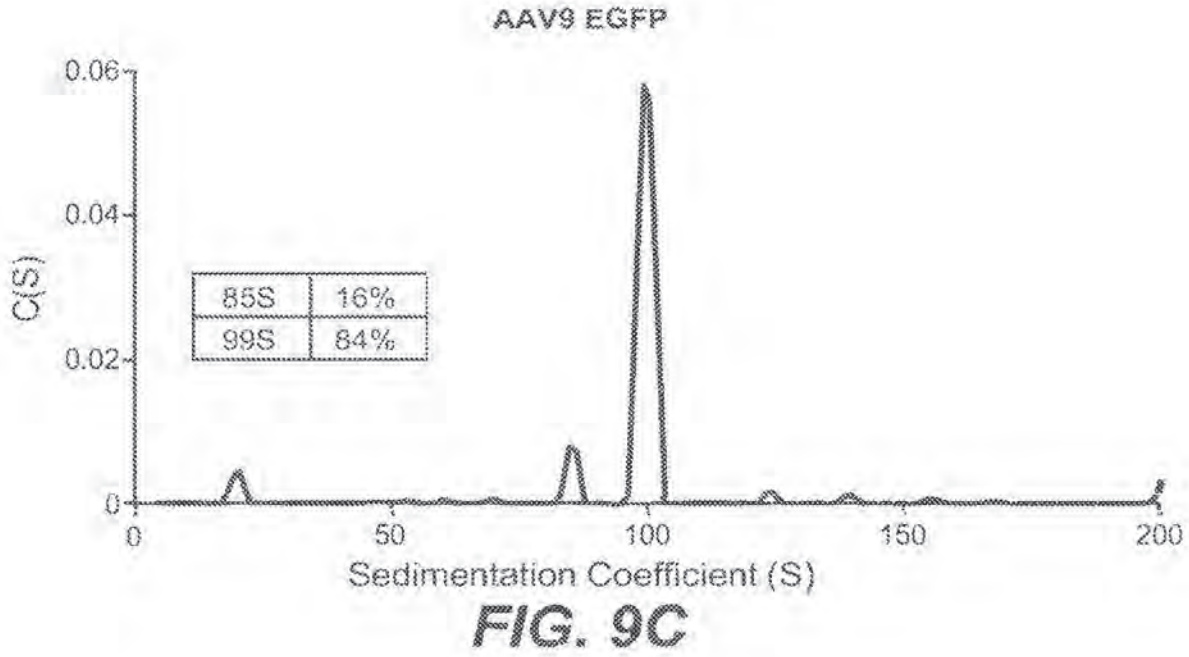

AAV9 EGFP
0.06
0.04
0.02
0
C(S)
85S 16%
99S 84%
0
50
100
150
200
Sedimentation Coefficient (S)

FIG. 9C

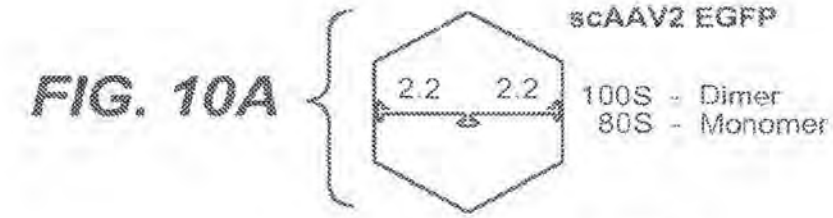


FIG. 10A

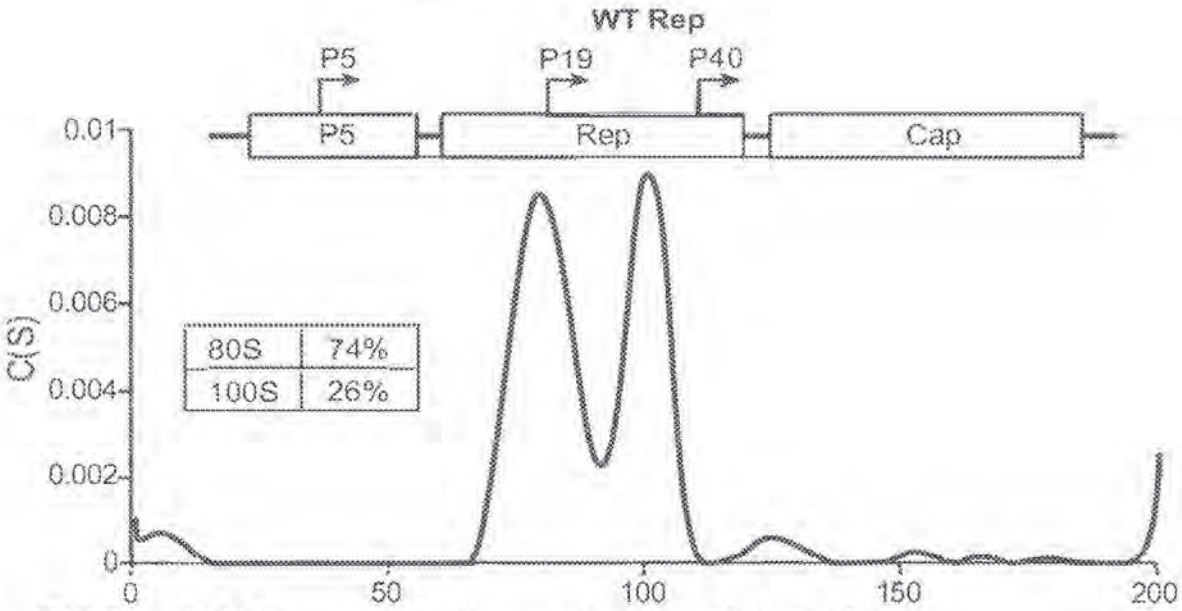


FIG. 10B

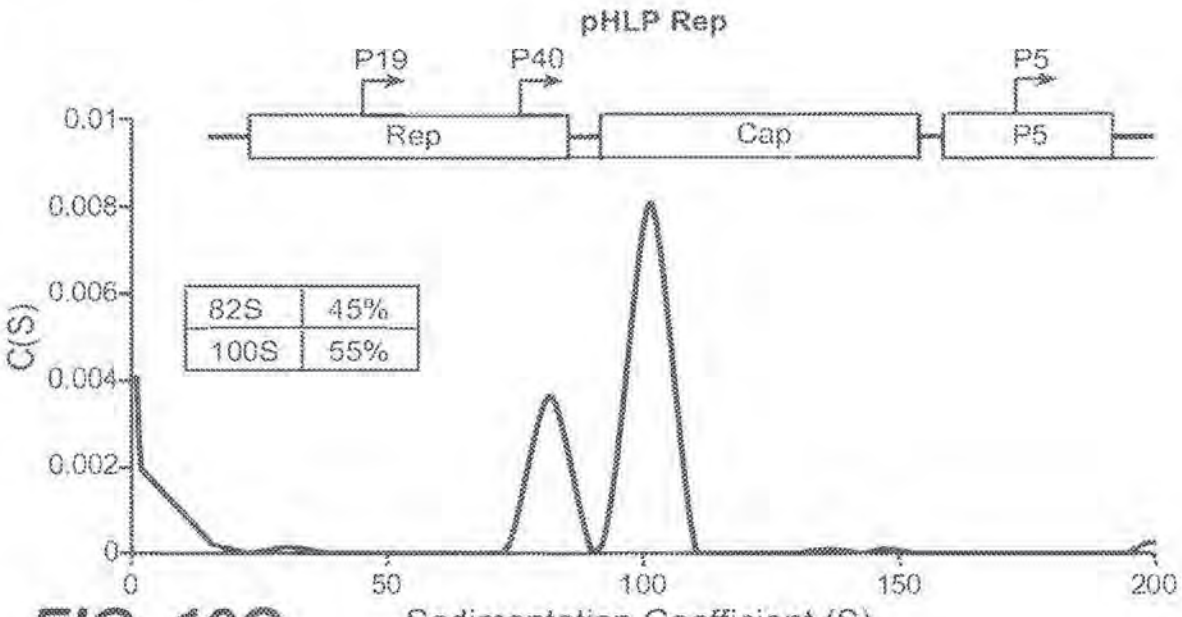


FIG. 10C

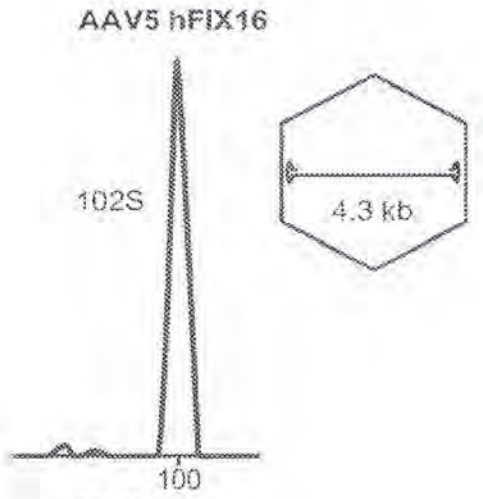


FIG. 11A

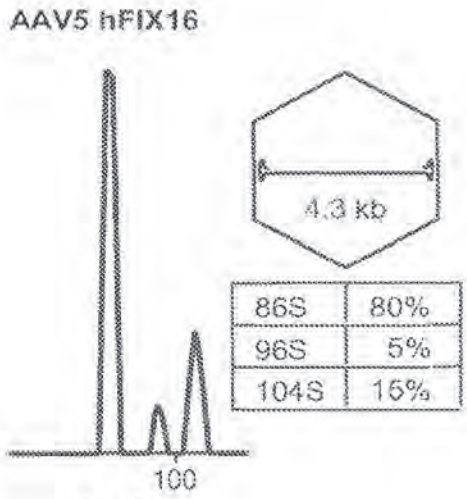


FIG. 11B

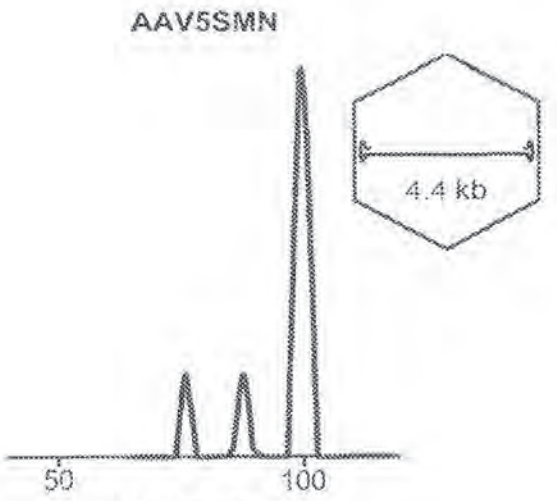


FIG. 11C

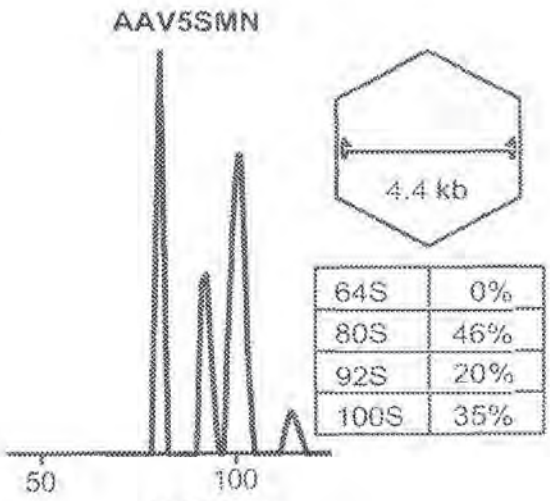


FIG. 11D

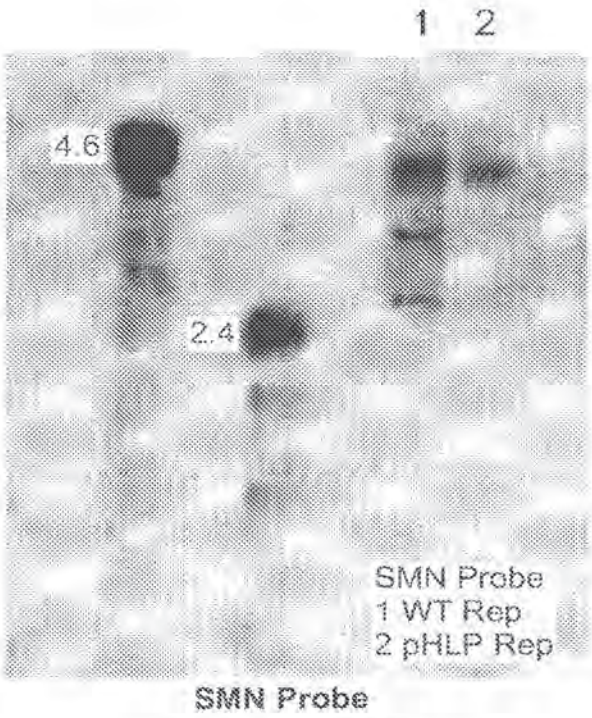


FIG. 12A

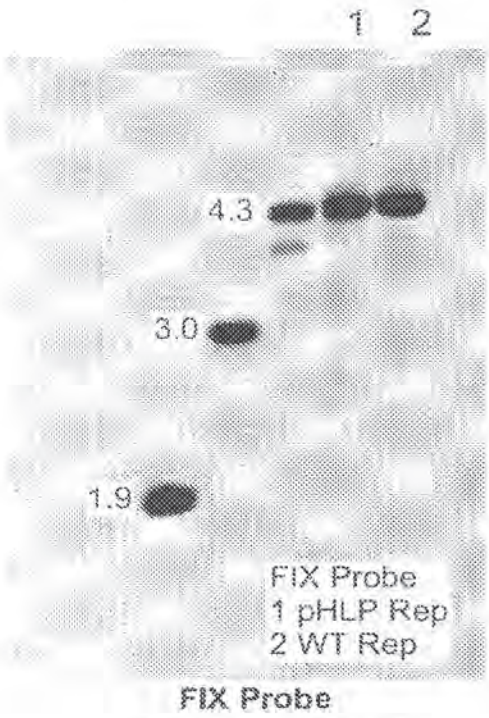


FIG. 12B

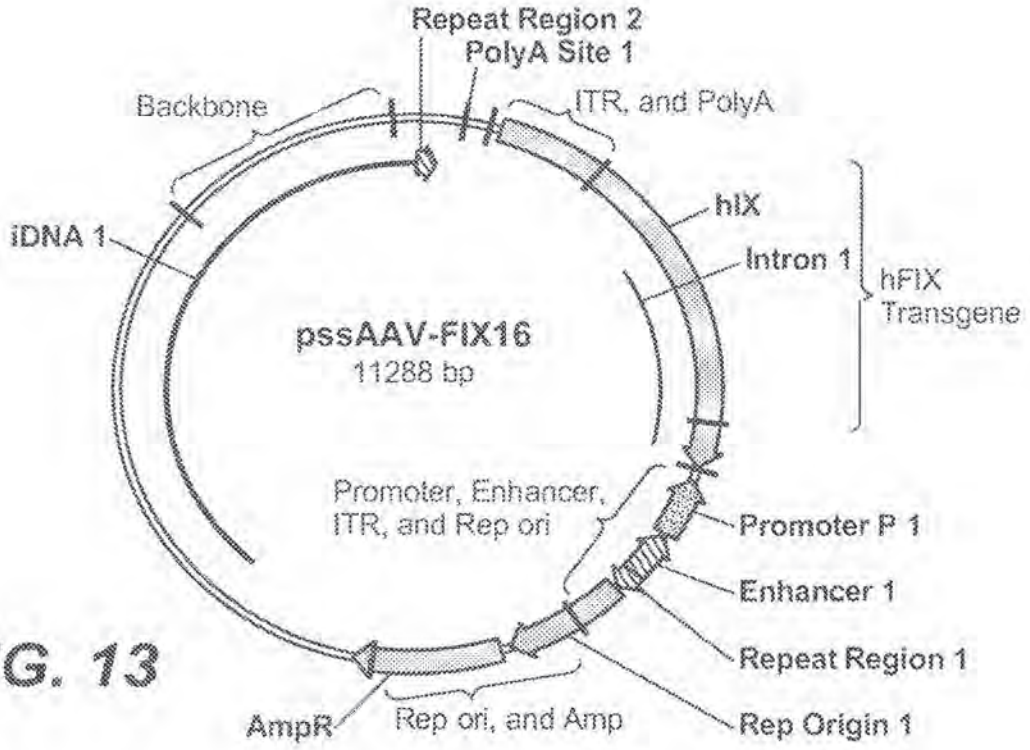
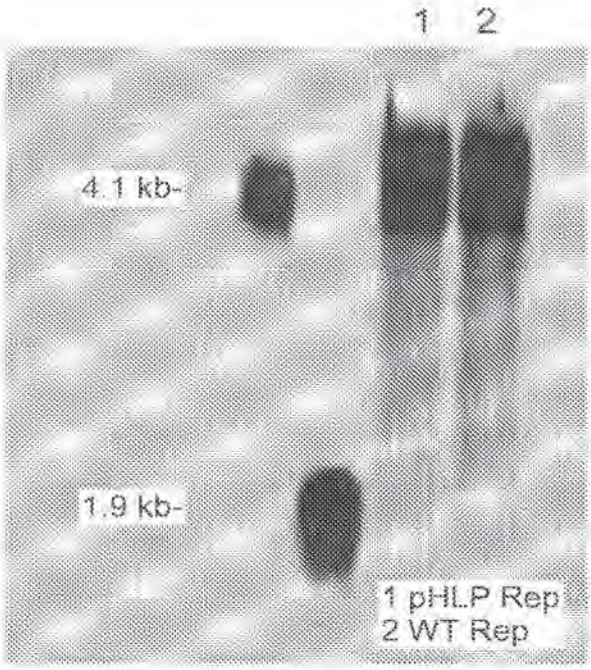
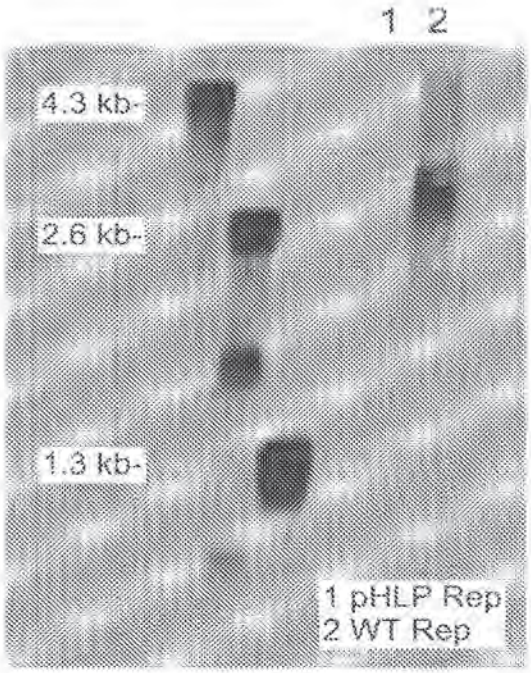


FIG. 13



Transgene

FIG. 14A



Rep ori & Amp

FIG. 14B

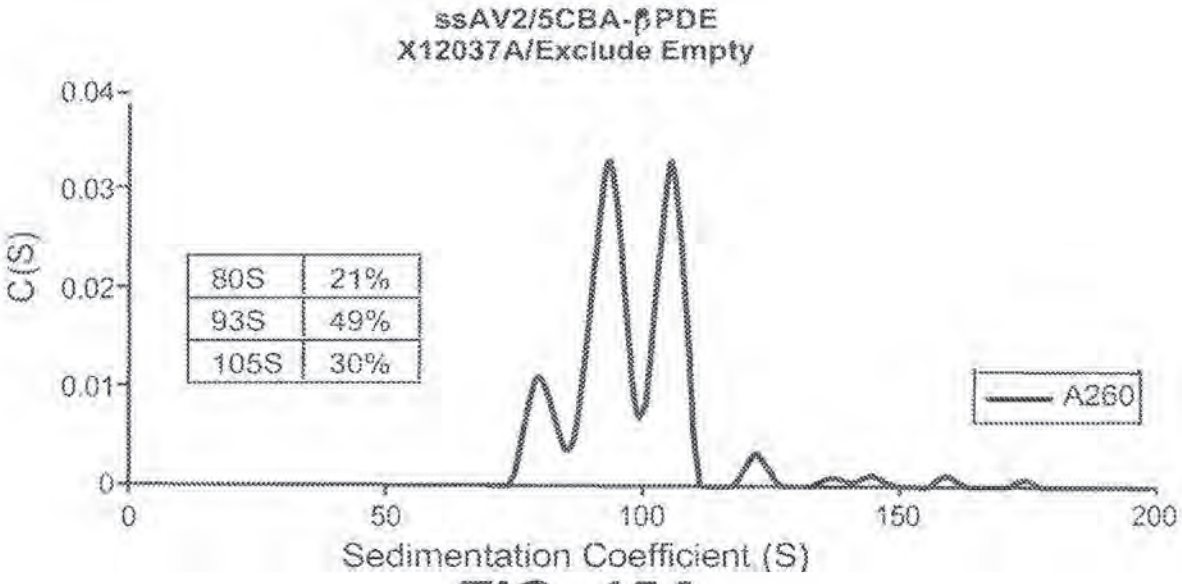


FIG. 15A

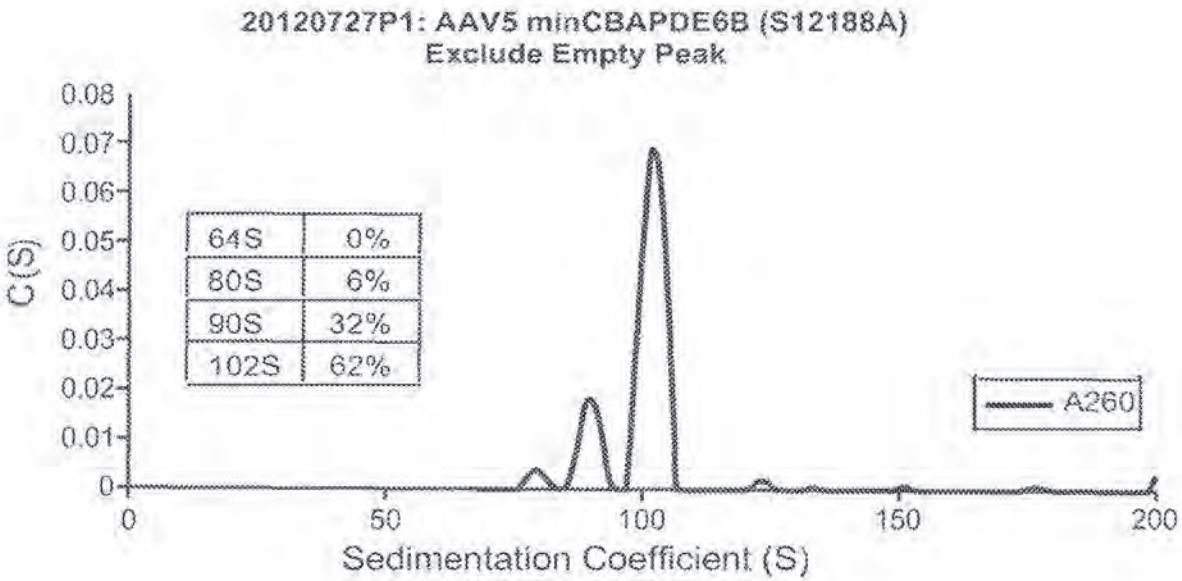


FIG. 15B

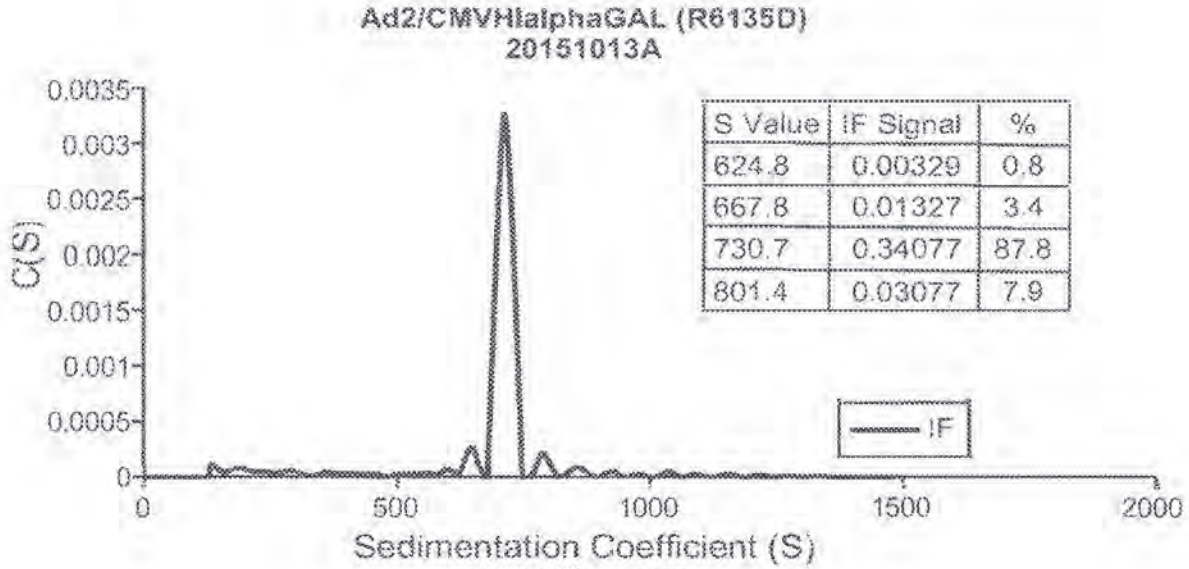

Ad2/CMVHIalphaGAL (R6135D)
20151013A
C(S)
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
500
1000
1500
2000
Sedimentation Coefficient (S)
IF
S Value | IF Signal | %
624.8 | 0.00329 | 0.8
667.8 | 0.01327 | 3.4
730.7 | 0.34077 | 87.8
801.4 | 0.03077 | 7.9

FIG. 16

ANALYTICAL ULTRACENTRIFUGATION FOR CHARACTERIZATION OF RECOMBINANT VIRAL PARTICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 18/188,176, filed Mar. 22, 2023, which is a continuation of U.S. patent application Ser. No. 16/547,144 (now U.S. Pat. No. 11,639,887), filed Aug. 21, 2019, which is a continuation of U.S. patent application Ser. No. 15/544,498 (now U.S. Pat. No. 10,429,288), which adopts the international filing date of Jan. 19, 2016, which is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2016/013947, filed Jan. 19, 2016, which claims priority to U.S. Provisional Application No. 62/105,714, filed Jan. 20, 2015, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

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

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).

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.

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

BRIEF SUMMARY OF THE INVENTION

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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/IBoV1 (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.

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 **30**, 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 recombi-

nant 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.

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.

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.

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

FIGS. **1**A and **1**B show that analytical ultracentrifugation (AUC) can be used to characterize recombinant viral vector particles. (FIG. **1**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"). (FIG. **1**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 (%).

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

FIGS. **3**A and **3**B show a comparison between interference and absorbance detection methods by AUC. (FIG. **3**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. (FIG. **3**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.

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.

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.

FIGS. **6**A, **6**B and **6**C shows that vector production by the producer cell line and triple transfection methods yields different vector preparations, as revealed by AUC analysis. (FIG. **6**A) A schematic of the AAV2-transgene 2 vector and its 3.4 kb genome. (FIG. **6**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. (FIG. **6**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.

FIGS. **7**A, **7**B and **7**C show that the AUC method may be used to monitor the quality and efficacy of vector purification. (FIG. **7**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. (FIG. **7**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. (FIG. **7**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.

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.

FIGS. **9**A, **9**B and **9**C show that assessment of capsid genome size using AUC data correlates with assessment of genome size by Southern blot. (FIG. **9**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. (FIG. **9**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. (FIG. **9**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 (%).

FIGS. **10**A, **10**B and **10**C show that the Rep/Cap promoter position affects genome packaging in recombinant viral vectors produced by the triple transfection method. (FIG. **10**A) A schematic of the self-complementary scAAV2 EGFP vector, with estimated sedimentation coefficients for the dimeric and monomeric genome species. (FIG. **10**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 (%). (FIG. **10**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 (%).

FIGS. **11**A, **11**B, **11**C and **11**D show that the Rep/Cap promoter position affects genome packaging in two additional AAV vectors. (FIGS. **11**A and **11**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," FIG. **11**B) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," FIG. **11**A). (FIGS. **11**C-**11**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," FIG. **11**D) or a p5 promoter downstream of the cap5 sequence ("pHLP Rep," FIG. **11**C).

FIGS. **12**A and **12**B reveal that Southern blot analysis correlates with AUC analysis but misses some fragmented genomes detectable by AUC. (FIG. **12**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. (FIG. **12**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.

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.

FIGS. **14**A and **14**B 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. **14**A) Southern blot analysis using an hFIX transgene-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes. (FIG. **14**B) Southern blot analysis using a Rep ori/AmpR-specific probe comparing pHLP Rep (lane 1) and WT Rep (lane 2) vector genomes.

FIGS. **15**A and **15**B show the fragmentation of oversized AAV vector genomes, as demonstrated by AUC analysis. (FIG. **15**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-PPDE). Peaks for detected species are labeled by observed sedimentation

coefficient (S) and relative abundance values (%). (FIG. **15**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 (%).

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

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.

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.

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.

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.

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.

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 recom-

binant viral preparation process) indicates an improvement in the production of recombinant viral particles.

I. General Techniques

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

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.

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.

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.

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).

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)".

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.

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."

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."

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."

"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.

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.

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.

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.

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).

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.

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.

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.

"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.

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.

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 104:1, at least about 10^6:1; or at least about 108:1 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 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).

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.

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.

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.

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.

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.

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.

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.

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).

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.

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."

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.

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

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, S A & Philo J S, (2007) *Anal. Biochem.*, 362:16-37.

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.

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.

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.

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.

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.

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:

$$\partial c/\partial t = D[(\partial^2 c/\partial r^2) + 1/r(\partial c/\partial r)] - s\omega^2[r(\partial c/\partial r) + 2c] \quad \text{Equation 1}$$

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.

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.

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.

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 1×10^{7} vg/mL, 2×10^{7} vg/mL, 3×10^{7} vg/mL, 4×10^{7} vg/mL, 5×10^{7} vg/mL, 6×10^{7} vg/mL, 7×10^{7} vg/mL, 8×10^{7} vg/mL, 9×10^{7} vg/mL, 1×10^{8} vg/mL, 2×10^{8} vg/mL, 3×10^{8} vg/mL, 4×10^{8} vg/mL, 5×10^{8} vg/mL, 6×10^{8} vg/mL, 7×10^{8} vg/mL, 8×10^{8} vg/mL, 9×10^{8} vg/mL, 1×10^{9} vg/mL, 2×10^{9} vg/mL, 3×10^{9} vg/mL, 4×10^{9} vg/mL, 5×10^{9} vg/mL, 6×10^{9} vg/mL, 7×10^{9} vg/mL, 8×10^{9} vg/mL, 9×10^{9} vg/mL, 1×10^{10} vg/mL, 2×10^{10} vg/mL, 3×10^{10} vg/mL, 4×10^{10} vg/mL, 5×10^{10} vg/mL, 6×10^{10} vg/mL, 7×10^{10} vg/mL, 8×10^{10} vg/mL, 9×10^{10} vg/mL, 1×10^{11} vg/mL, 2×10^{11} vg/mL, 3×10^{11} vg/mL, 4×10^{11} vg/mL, 5×10^{11} vg/mL, 6×10^{11} vg/mL, 7×10^{11} vg/mL, 8×10^{11} vg/mL, 9×10^{11} vg/mL, 1×10^{12} vg/mL, 2×10^{12} vg/mL, 3×10^{12} vg/mL, 4×10^{12} vg/mL, 5×10^{12} vg/mL, 6×10^{12} vg/mL, 7×10^{12} vg/mL, 8×10^{12} vg/mL, 9×10^{12} vg/mL, 1×10^{13} vg/mL, 2×10^{13} vg/mL, 3×10^{13} vg/mL, 4×10^{13} vg/mL, 5×10^{13} vg/mL, 6×10^{13} vg/mL, 7×10^{13} vg/mL, 8×10^{13} vg/mL, 9×10^{13} vg/mL. In some embodiments, the viral particles are concentrated to of about 1×10^{7} vg/mL to about 1×10^{13} vg/mL, about 1×10^{8} vg/mL to about 1×10^{13} vg/mL, about 1×10^{9} vg/mL to about 1×10^{13} vg/mL, about 1×10^{10} vg/mL to about 1×10^{13} vg/mL, about 1×10^{11} vg/mL to about 1×10^{13} vg/mL, about 1×10^{12} vg/mL to about 1×10^{13} vg/mL, about 1×10^{7} vg/mL to about 1×10^{12} vg/mL, about 1×10^{8} vg/mL to about 1×10^{12} vg/mL, about 1×10^{9} vg/mL to about 1×10^{12} vg/mL, about 1×10^{10} vg/mL to about 1×10^{12} vg/mL, about 1×10^{11} vg/mL to about 1×10^{12} vg/mL, about 1×10^{7} vg/mL to about 1×10^{11} vg/mL, about 1×10^{8} vg/mL to about 1×10^{11} vg/mL, about 1×10^{9} vg/mL to about 1×10^{11} vg/mL, about 1×10^{10} vg/mL to about 1×10^{11} vg/mL, about 1×10^{7} vg/mL to about 1×10^{10} vg/mL, about 1×10^{8} vg/mL to about 1×10^{10} vg/mL, about 1×10^{9} vg/mL to about 1×10^{10} vg/mL, about 1×10^{7} vg/mL to about 1×10^{9} vg/mL, about 1×10^{8} vg/mL to about 1×10^{9} vg/mL, or about 1×10^{7} vg/mL to about 1×10^{8} vg/mL.

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, W F et al., (2014) *Herpes Simplex Virus Methods in Molecular Biology* 1144:63-79.

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 260 nm 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.

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.

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 (2^{nd}) 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 9005, 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 9005, 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.

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 ($\epsilon_{260/capsid}$=30.72e6) 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.

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.

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 par-

ticles 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.

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.

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.

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.

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.

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.

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.

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	260 nm 280 nm 230 nm	260 nm 280 nm 230 nm	260 nm 280 nm 230 nm	260 nm 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	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

TABLE 1-continued

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

	AAV	Ad	Lentivirus	HSV
Temperature ranges	IF only: collect every 10-60 seconds (10-60 second delay) 4° C.-20° C.	4° C.-20° C.	4° C. -20° C.	4° C.-20° C.

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.

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.

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.

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, recombi-

nants, etc.). In some embodiments the variant genomes include genomes that are smaller and larger than the intact recombinant viral genome.

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.

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.

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.

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

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).

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).

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.

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

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 U.S. Pat. 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 non-coding 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.

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 (~950 Å), 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.

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, AdHu 11, 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.

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.

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 (RD 114), 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 RD 114 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.

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.

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

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, J E 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. Pat. No. 6,566,118, and Sf-900 II SFM media as described in U.S. Pat. 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).

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.

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 U.S. Pat. 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.

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.

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. Pat. 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.

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.

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.

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, W F et al., (2014) Herpes Simplex Virus Methods in Molecular Biology 1144:63-79).

VI. Purification of rAAV Vectors

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, AAV 11, 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.

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 m 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 m or greater pore size known in the art.

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.

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; U.S. Pat. Nos. 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, W F et al., (2014) *Herpes Simplex Virus Methods in Molecular Biology* 1144:63-79.

EXAMPLES

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

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, B J, 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.

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

In order to support accurate AUC assessment, vector product (AAV2-transgene 2) was highly purified, suitably buffered, and concentrated to greater than 5×10^{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 260 nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260 nm 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 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

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 (2^{nd}) 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)

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

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

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

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

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

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 260 nm along a centrifugal field at defined time intervals. FIG. **1**A 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. **1**A).

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. **1**B). 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 ($Abs_{260\ nm}$)	$\varepsilon_{260\ nm}$	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	

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.

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. **2**A) or genome containing particles (FIG. **2**B). 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.

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.

		Full Capsid, Peak 3	
AUC Run	Empty Capsid, Peak 1 (S)	% 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

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.

Pure populations of empty and genome-containing AAV2 capsids were mixed at a 1:1 ratio and analyzed by AUC using both interference (FIG. **3**A) and absorbance detection methods (FIG. **3**B). Interference detection revealed two populations of AAV capsids at the approximate expected ratios of 43% empty and 57% genome-containing (FIG. **3**A). 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 FIGS. **3**A and **3**B). The data generated by both methods are compared in Table 4. The ratio of absorbance signal to interference signal ($A_{260\ nm}$/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.

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

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

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.

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. **6**A shows a schematic of this AAV2 vector genome.

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. **6**B). 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. **6**C), with 11% of capsids containing a fragmented genome (76S and 84S species) and only 8% of capsids having a full genome (94S).

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

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. **7**A). 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. **7**A).

As shown in FIG. **7**B, 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. **7**C) 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

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.

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) (y)	Genome size (# NT) (x)	MW	Extinction coefficient (260 nm)
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

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 (AAV2/9minCBAE-GFP; see schematic in FIG. **9**A). 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. **9**A). 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. **9**A), 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).

FIG. **9**B 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. **9**A. 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. **9**C). 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. **9**B). 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

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

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. **10**A) was produced using one of two methods. In the first method (FIG. **10**B), 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. **10**C), 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. **10**A).

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. **10**C). 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. **10**B). 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.

A single stranded AAV5 Factor IX vector, AAV5FIX, (FIG. **11**A-B) and a single stranded AAV5hSMN vector (FIG. **11**C-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. **11**A). 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. **11**B). 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. **11**B).

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. **11**C). 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. **11**D). These results confirm a significant difference in genome packaging induced by shifting the P5 promoter of the helper plasmid using two additional AAV vectors.

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. **12**A, 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. **12**A, 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. **12**B, lane 2). However, AUC analysis of this AAV5FIX vector (FIG. **11**B) showed that 80% of the capsids contained a fragmented genome (~3000 nucleotides), which were undetected by the FIX probe.

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. **14**A, both vector preparations (pHLP rep, lane 1; WT rep, lane 2) contained the hFIX transgene. However, FIG. **14**B lane 2confirms that the vector genome species sedimenting at 86S observed in the WT Rep preparation (FIG. **11**B) was a ~3kb fragment. Moreover, this species reacted with an Amp^R specific probe (FIG. **14**B, 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. **14**B, lane 1).

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.

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 P-phosphodiesterase transgene was packaged as an oversized genome of 5.4kb (FIG. **15**A) or as a wild type size genome of 4.6kb (FIG. **15**B). To generate the 4.6kb genome, the

CBA promoter was truncated by reducing the size of the intron as previously reported (Gray, S J et al., (2011) *Hum. Gene Ther.* 22(9):1143-1153).

As shown in FIG. **15**A, 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. **15**B). 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.

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

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

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 260 nm (OD_{260}) by spectrophotometric methods. To generate reproducible and consistent AUC data, sample adjustments were made to target concentration by optical density measurement at 260 nm 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

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 (2^{nd}) 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

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.

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.

What is claimed is:

1. A method of determining the size of one or more fragmented genomes in a preparation of viral particles comprising recombinant adeno-associated viral (rAAV) vectors encapsidated into viral capsids, said method comprising:

(i) subjecting the preparation to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate one or more sedimenting boundaries, wherein the boundary sedimentation velocity is from about 3,000 rpm to about 20,000 rpm;

(ii) measuring the rate of movement or migration of the one or more sedimenting boundaries, wherein movement or migration of the viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable viral particle, and wherein one or more of the viral particles comprise a fragmented genome, and determining the sedimentation coefficients of the viral particles comprising one or more of the fragmented genomes in the preparation; and

(iii) determining the size of the one or more fragmented genomes as a function of the sedimentation coefficients of the viral particles comprising the one or more fragmented genomes.

2. The method of claim **1**, wherein step (iii) comprises comparing the sedimentation coefficients of the one or more viral particles comprising the fragmented genomes with a standard curve generated from sedimentation coefficients of viral particles comprising recombinant AAV genomes of known nucleotide sizes, thereby determining the size of the one or more fragmented genomes.

3. The method of claim **1**, wherein the boundary sedimentation velocity is from about 10,000 rpm to about 20,000 rpm.

4. The method of claim **1**, wherein the boundary sedimentation velocity is from about 15,000 rpm to about 20,000 rpm.

5. The method claim **4**, wherein the boundary sedimentation is performed at a temperature of from about 4° C.to about 20° C.

6. The method of claim **5**, wherein the boundary sedimentation is performed at a temperature of about 4° C.

7. The method of claim **1**, wherein the fragmented genomes are sub-genomic DNA molecules.

8. The method of claim **1**, wherein the centrifugation is run from about 0.5 hours to about 2 hours.

9. The method of claim **8**, wherein the centrifugation is run from about 1 hours to about 2 hours.

10. The method of claim **8**, wherein the centrifugation is run for about 1 hour.

11. The method of claim **8**, wherein the centrifugation is run for about 1.2 hours.

12. The method of claim **1**, wherein the total concentration of viral particles in the AAV vector preparation prior to step (i) is greater than 5×10^{11} vg/mL.

13. The method of claim **1**, wherein the total concentration of viral particles in the AAV vector preparation prior to step (i) is from about 1×10^{11} vg/mL to about 1×10^{13} vg/mL.

14. The method of claim **1**, further comprising quantifying of the viral particles comprising the one or more fragmented genomes in the preparation.

15. The method of claim **1**, comprising determining the size of all of the fragmented genomes in the preparation.

16. The method of claim **15**, further comprising quantifying all of the fragmented genomes in the preparation.

17. The method of claim **1**, wherein the preparation comprises AAV9 capsid proteins.

18. The method of claim **17**, wherein the preparation further comprises at least one AAV2 ITR.

19. The method of claim **16**, wherein the preparation comprises self-complementary AAV viral genomes.

20. A method of determining the molar concentrations of each species of individual viral particles in a heterogeneous mixture of viral particles comprising recombinant adeno-associated viral (rAAV) vectors encapsidated into viral capsids, said method comprising:

(i) subjecting the heterogeneous mixture of viral particles to analytical ultracentrifugation under boundary sedimentation velocity conditions to generate sedimenting boundaries, wherein the boundary sedimentation velocity is from about 3,000 rpm to about 20,000 rpm;

(ii) measuring the rate of movement or migration of the sedimenting boundaries, wherein movement or migration of each species of the individual viral particles in the heterogeneous mixture of viral particles results in distinct sedimenting boundaries, each distinct sedimenting boundary corresponding to a resolvable species of viral particle, and wherein the heterogeneous mixture of viral particles comprises full genomes, fragmented genomes and empty capsids without genome;

(iii) determining the sedimentation coefficients of each species of the individual viral particles in the heterogeneous mixture of viral particles; and

(iv) quantifying the molar concentration of each species of the individual viral particles in the heterogeneous mixture of viral particles.

21. The method of claim **20**, further comprising determining the size of each species of the individual viral particles after step (iii) and prior to step (iv) by comparing the sedimentation coefficients of each species of the individual viral particles with a standard curve generated from sedimentation coefficients of viral particles comprising recombinant AAV genomes of known nucleotide sizes.

22. The method of claim **20**, wherein the boundary sedimentation velocity is from about 10,000 rpm to about 20,000 rpm.

23. The method of claim **20**, wherein the boundary sedimentation velocity is from about 15,000 rpm to about 20,000 rpm.

24. The method of claim **20**, wherein the boundary sedimentation is performed at a temperature of about 4° C.

25. The method of claim **20**, wherein the centrifugation is run from about 0.5 hours to about 2 hours.

26. The method of claim **20**, wherein the total concentration of viral particles in the heterogeneous mixture prior to step (i) is greater than 5×10^{11} vg/mL.

27. The method of claim **20**, wherein the total concentration of viral particles in the heterogeneous mixture prior to step (i) is from about 1×10^{11} vg/mL to about 1×10^{13} vg/mL.

28. The method of claim **20**, wherein the viral particles comprise AAV9 capsid proteins.

29. The method of claim 28, wherein the viral particles comprise at least one AAV2 ITR.

30. The method of claim 20, further comprising determining the relative percentage of each species of the individual viral particles in the heterogeneous mixture of viral particles.

* * * * *

Exhibit Q

US011698377B2

(12) United States Patent

Jin et al.

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(45) Date of Patent: Jul. 11, 2023

(54) **METHODS FOR DETECTING AAV**

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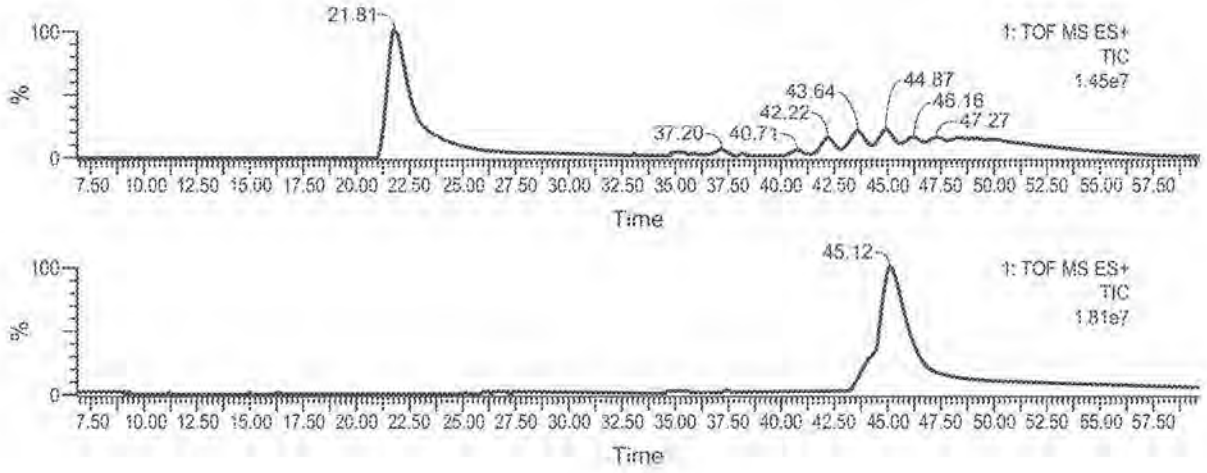
(57) **ABSTRACT**

Provided herein are methods for determining the serotype of a virus particle and/or or determining the heterogeneity of a virus particle (e.g., an AAV particle). In other embodiments, the invention provides methods to determine the heterogeneity of AAV particles. In some aspects, the invention provides viral particles (e.g., rAAV particles) with improved stability and/or improved transduction efficiency by increasing the acetylation and/or deamidation of capsid proteins.

20 Claims, 27 Drawing Sheets

Specification includes a Sequence Listing.



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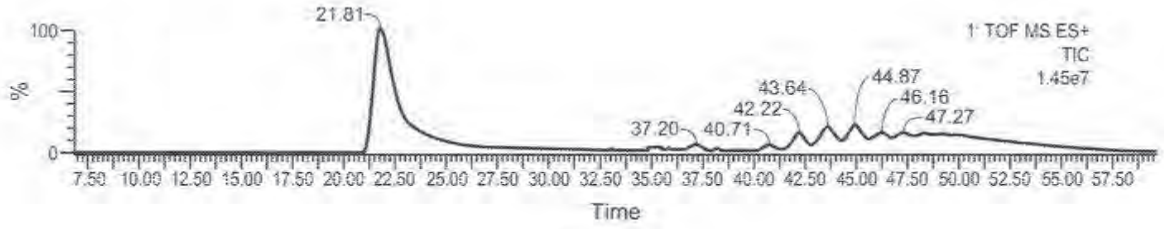

21.81
37.20
40.71
42.22
43.64
44.87
46.16
47.27
1: TOF MS ES+
TIC
1.45e7
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1A

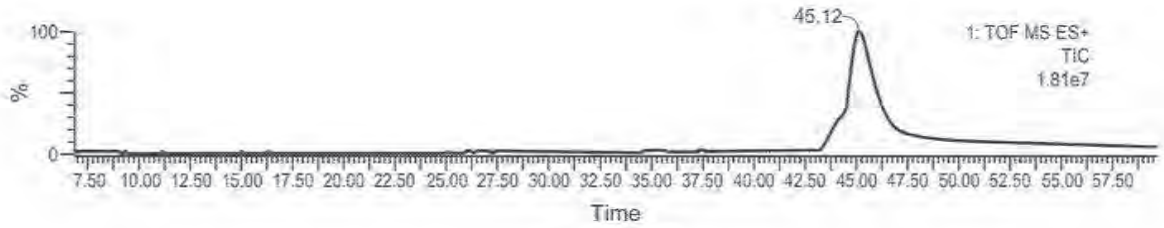

45.12
1: TOF MS ES+
TIC
1.81e7
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1B

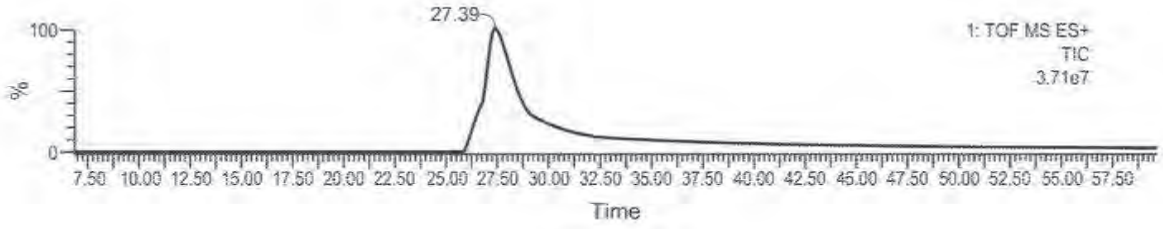

27.39
100
%
0
1: TOF MS ES+
TIC
3.71e7
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1C

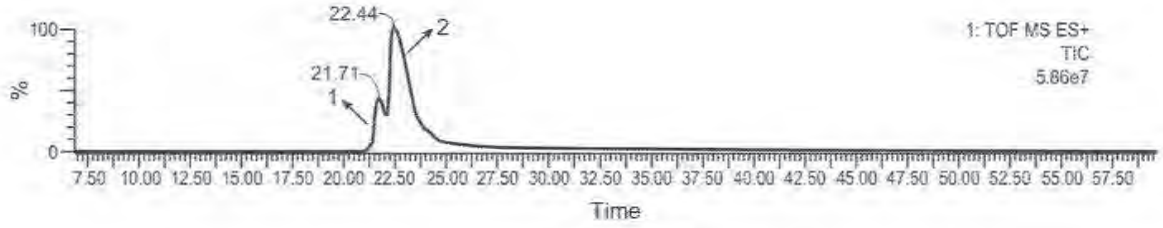

22.44
2
21.71
1
100
%
0
1: TOF MS ES+
TIC
5.66e7
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1D

FIG. 2A

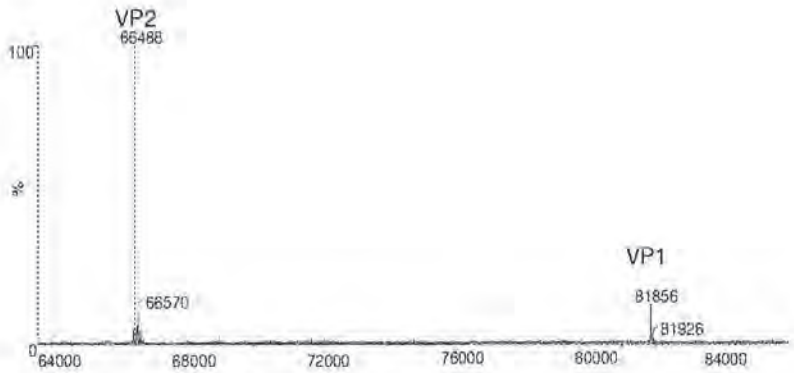
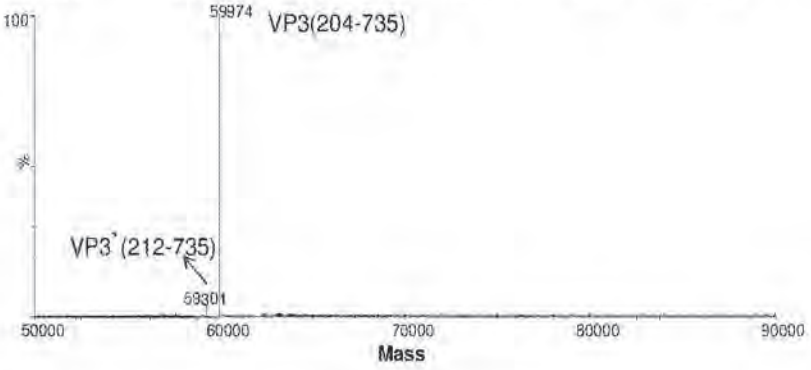

VP2
66488
100
%
VP1
66570
81856
81826
0
64000
68000
72000
76000
80000
84000

FIG. 2B


100
%
59974
VP3(204-735)
VP3*(212-735)
59301
50000
60000
70000
80000
90000
Mass

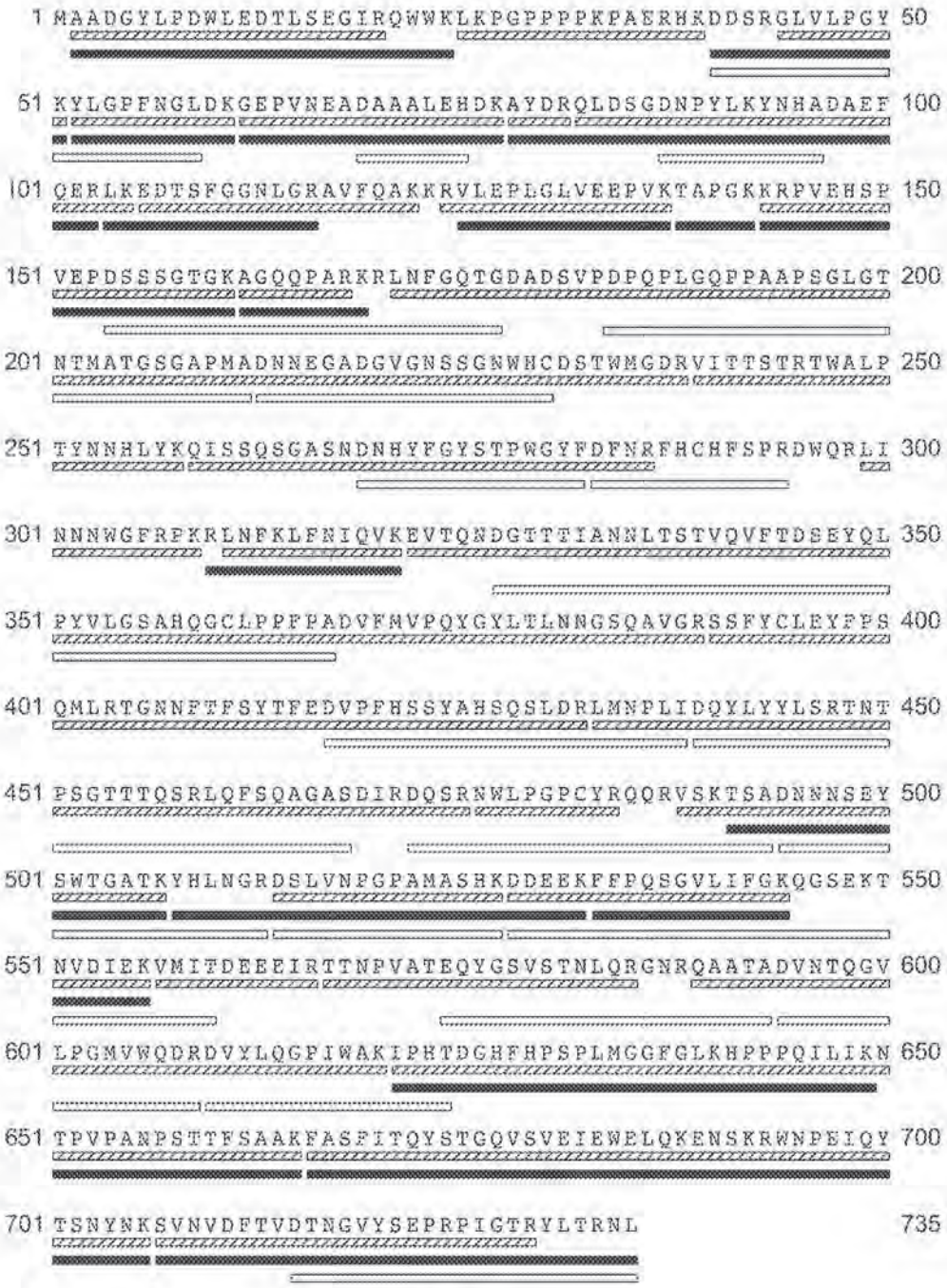

1 MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY 50
51 KYLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEF 100
101 QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP 150
151 VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT 200
201 NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP 250
251 TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI 300
301 NNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQL 350
351 PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS 400
401 QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT 450
451 PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY 500
501 SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT 550
551 NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV 600
601 LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN 650
651 TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY 700
701 TSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL 735

FIG. 3

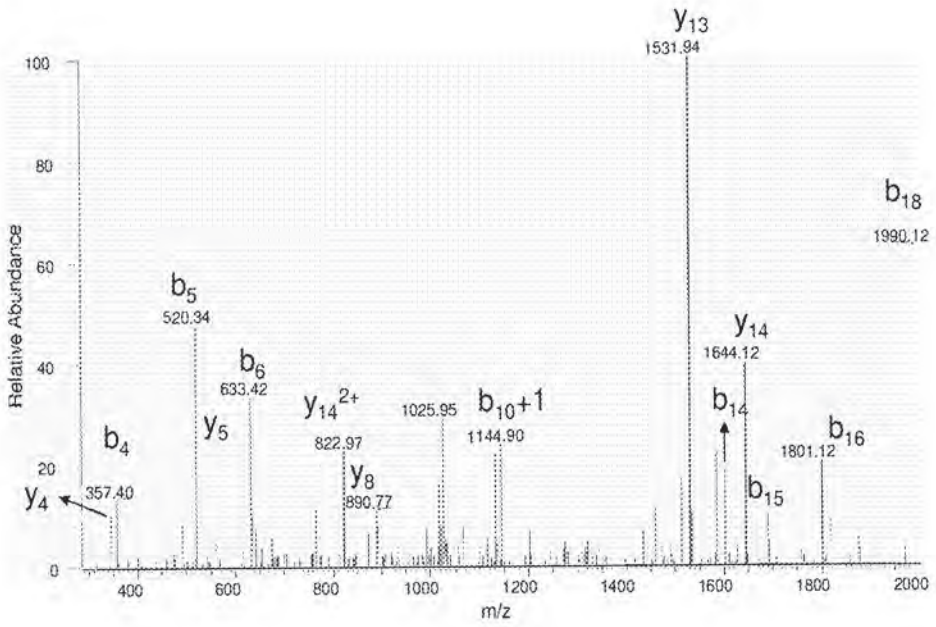

y13
1531.94
b18
1990.12
b5
520.34
y14
1644.12
b6
633.42
y14 2+
822.97
1025.95
b10+1
1144.90
b14
b16
1801.12
y5
b4
y8
890.77
y4
357.40
b15
Relative Abundance
m/z
100
80
60
40
20
0
400
600
800
1000
1200
1400
1600
1800
2000

FIG. 4A

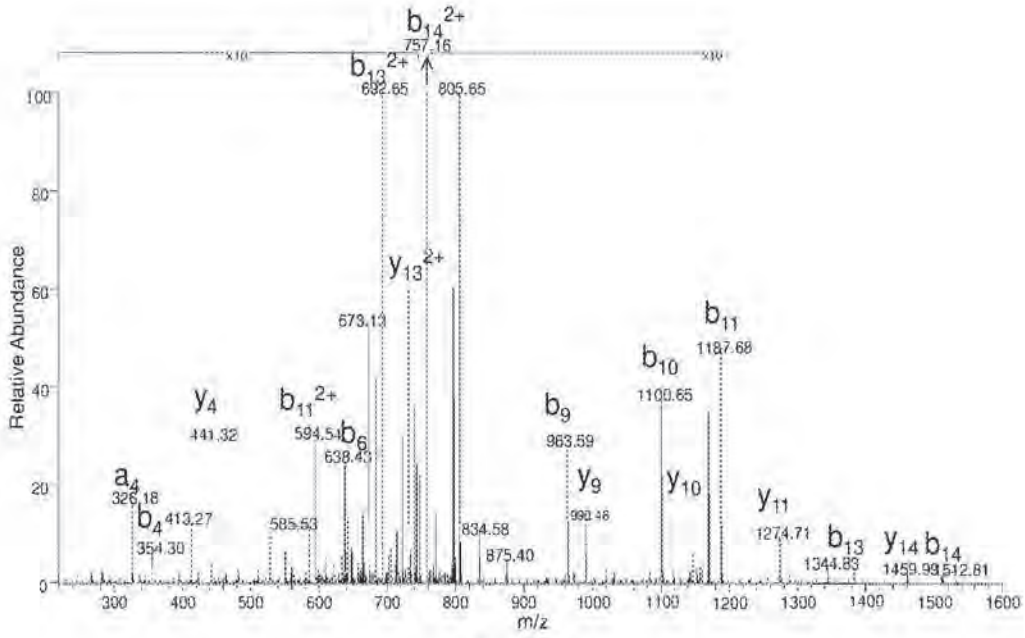

Relative Abundance
m/z
b14 2+
757.16
b13 2+
682.65
805.65
y13 2+
673.13
b11
1187.68
b10
1100.65
y4
441.32
b11 2+
594.54
b6
638.43
b9
963.59
a4
326.18
b4
413.27
354.30
585.53
834.58
875.40
y9
992.48
y10
y11
1274.71
b13
1344.83
y14
b14
1459.99
512.81

FIG. 4B

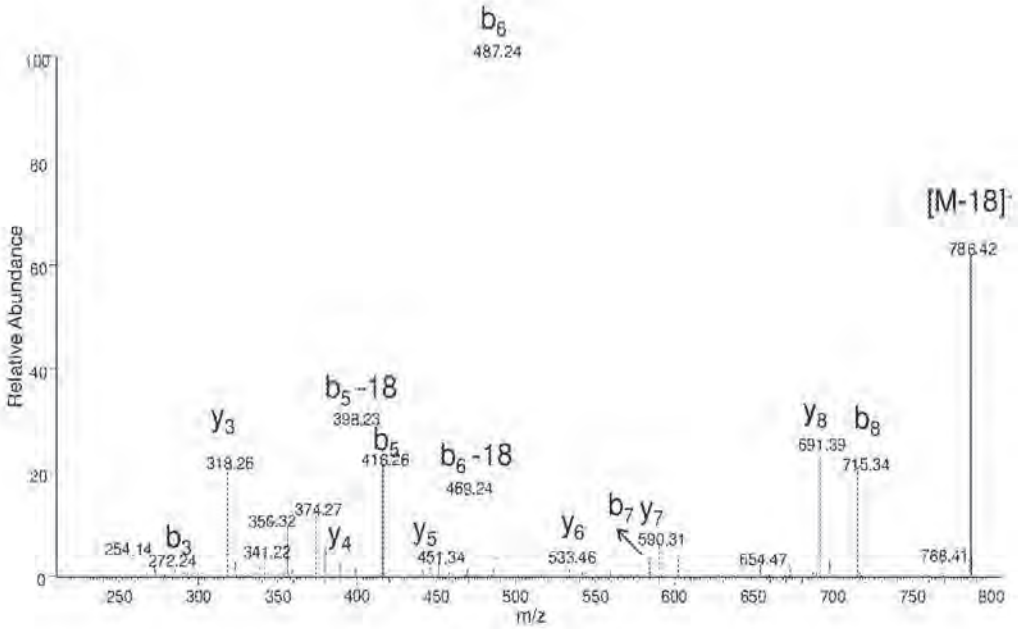

b6
487.24
[M-18]
786.42
Relative Abundance
100
80
60
40
20
0
b5-18
398.23
y3
319.26
b5
416.26
b6-18
469.24
y8
591.39
b8
715.34
b7 y7
590.31
y6
533.46
y5
451.34
y4
374.27
356.32
341.22
b3
272.24
254.14
654.47
768.41
250
300
350
400
450
500
550
600
650
700
750
800
m/z

FIG. 4C

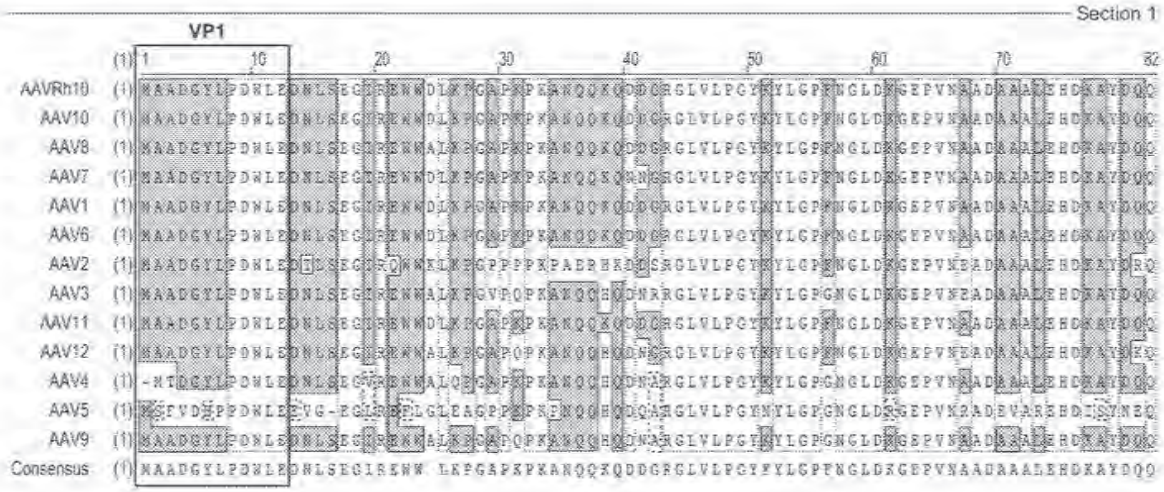

Section 1
VP1
AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 5A

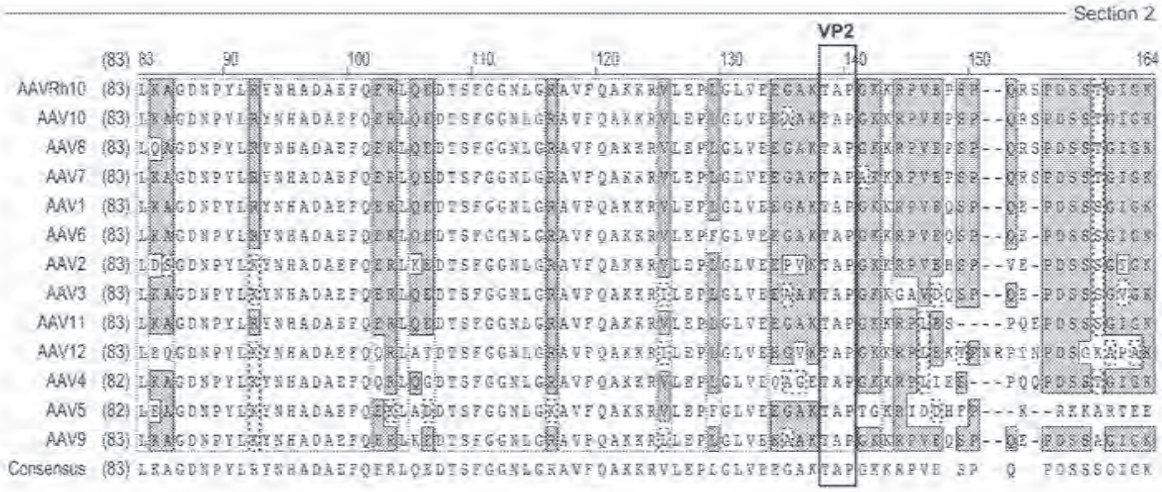

Section 2
VP2
(83) 83 90 100 110 120 130 140 150 164
AAVRh10 (83)
AAV10 (83)
AAV8 (83)
AAV7 (83)
AAV1 (83)
AAV6 (83)
AAV2 (83)
AAV3 (83)
AAV11 (83)
AAV12 (83)
AAV4 (82)
AAV5 (82)
AAV9 (83)
Consensus (83)

FIG. 5B

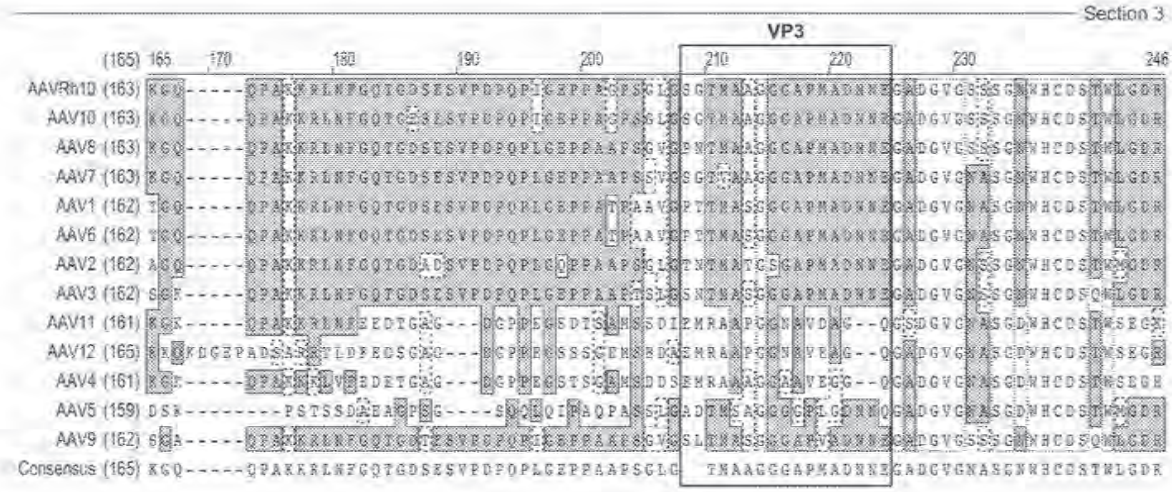

Section 3
VP3
(165) 165 170 180 190 200 210 220 230 246
AAVRh10 (163)
AAV10 (163)
AAV8 (163)
AAV7 (163)
AAV1 (162)
AAV6 (162)
AAV2 (162)
AAV3 (162)
AAV11 (161)
AAV12 (166)
AAV4 (161)
AAV5 (159)
AAV9 (162)
Consensus (165)

FIG. 5C

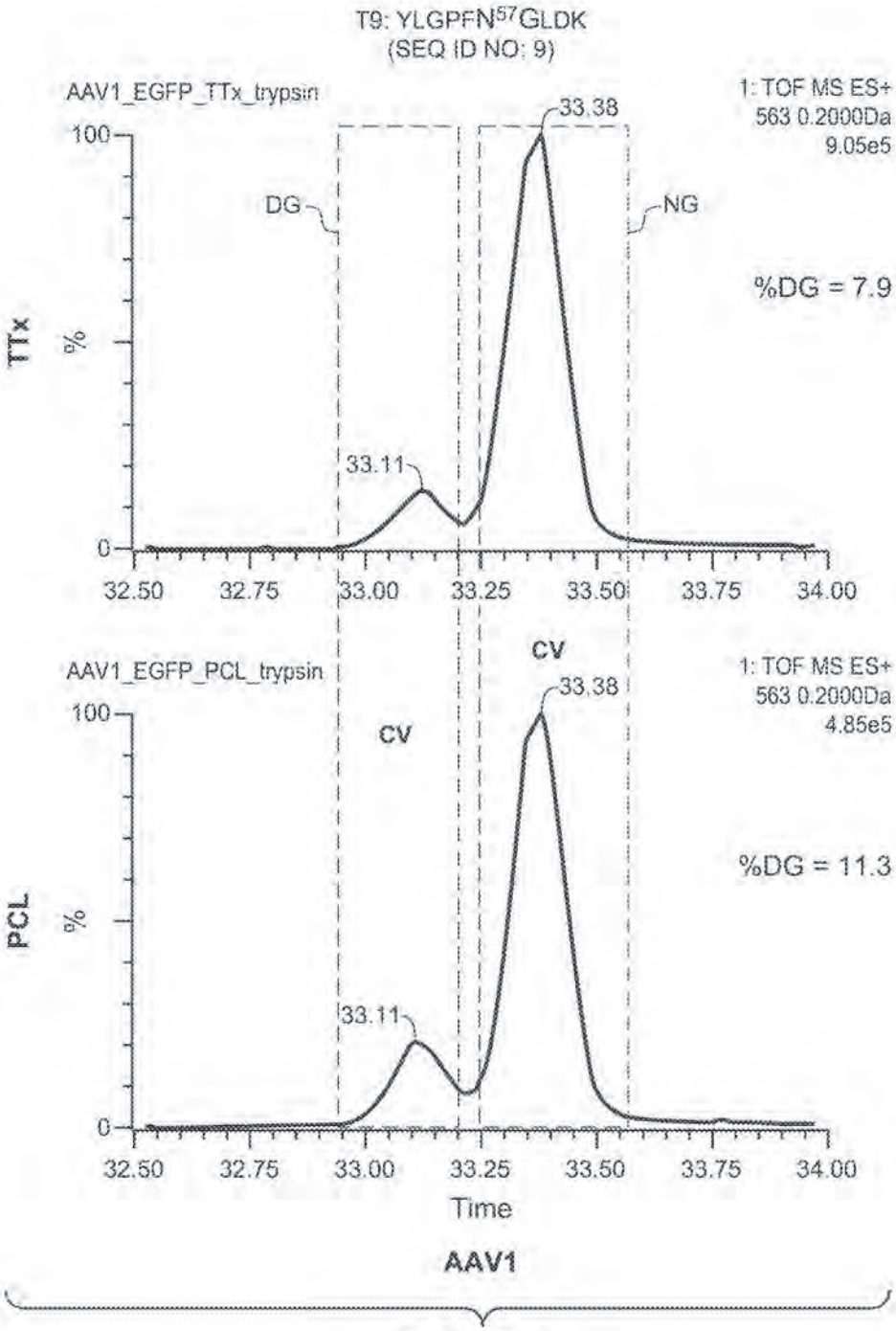

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
9.05e5
33.38
DG
NG
%DG = 7.9
TTx
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV1_EGFP_PCL_trypsin
CV
CV
1: TOF MS ES+
563 0.2000Da
4.85e5
33.38
%DG = 11.3
PCL
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
Time
AAV1

FIG. 6A

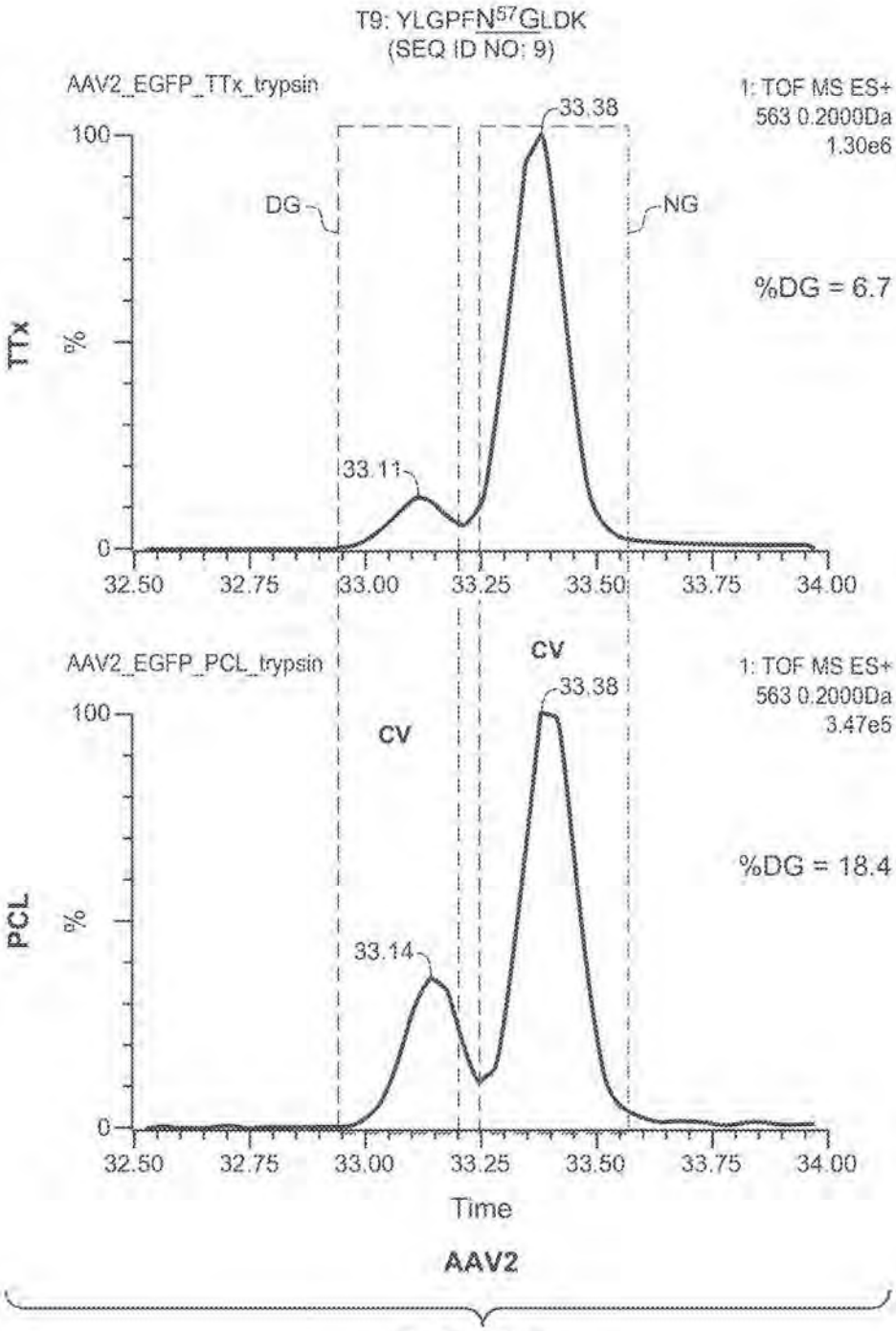

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
1.30e6
33.38
DG
NG
%DG = 6.7
TTx
%
33.11
100
0
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV2_EGFP_PCL_trypsin
CV
CV
1: TOF MS ES+
563 0.2000Da
3.47e5
33.38
%DG = 18.4
PCL
%
33.14
Time
AAV2

FIG. 6B

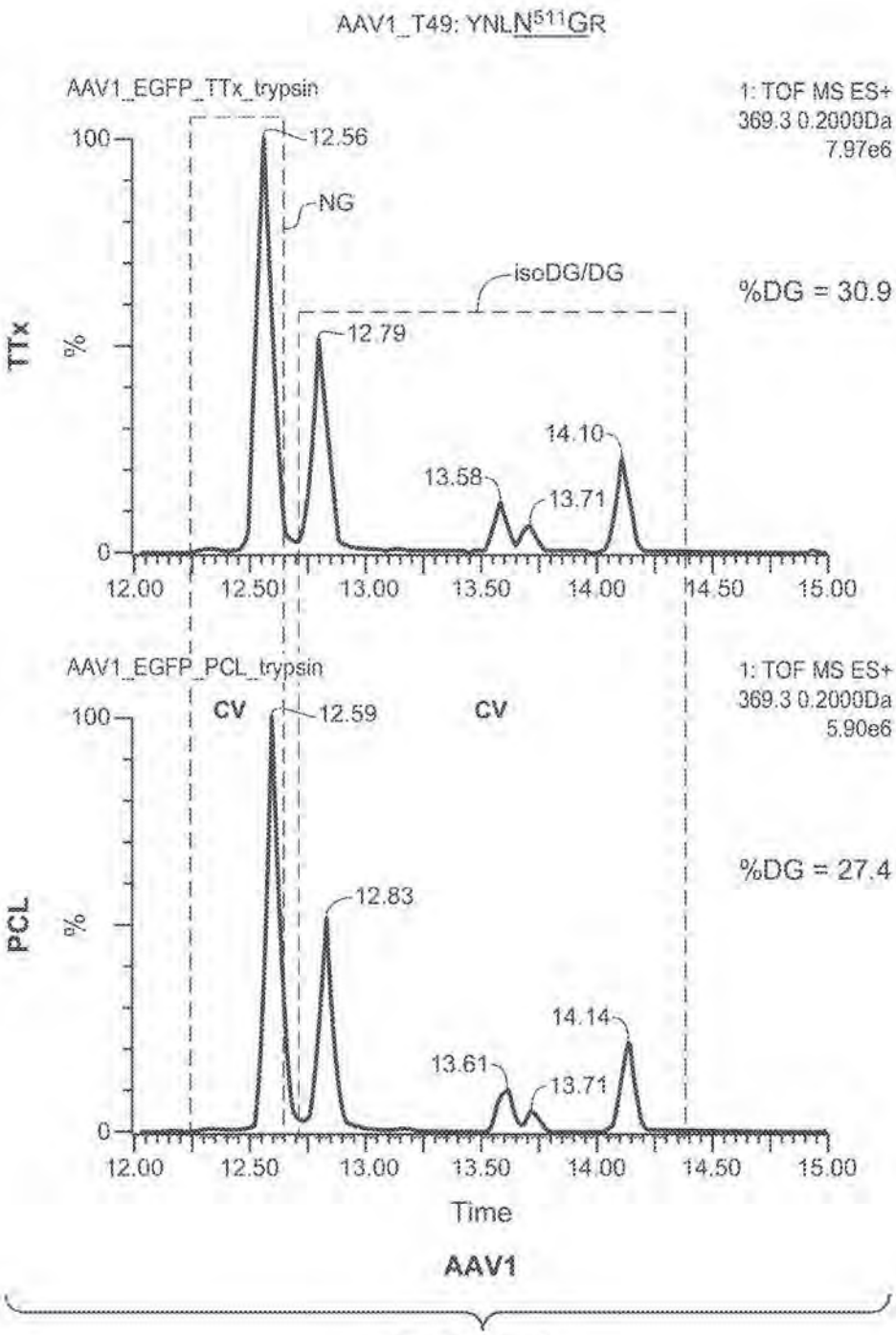

AAV1_T49: YNLN511GR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
369.3 0.2000Da
7.97e6
100
12.56
NG
isoDG/DG
%DG = 30.9
TTx
%
12.79
14.10
13.58
13.71
0
12.00
12.50
13.00
13.50
14.00
14.50
15.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
369.3 0.2000Da
5.90e6
CV
12.59
CV
100
%DG = 27.4
PCL
%
12.83
14.14
13.61
13.71
0
12.00
12.50
13.00
13.50
14.00
14.50
15.00
Time
AAV1

FIG. 7A

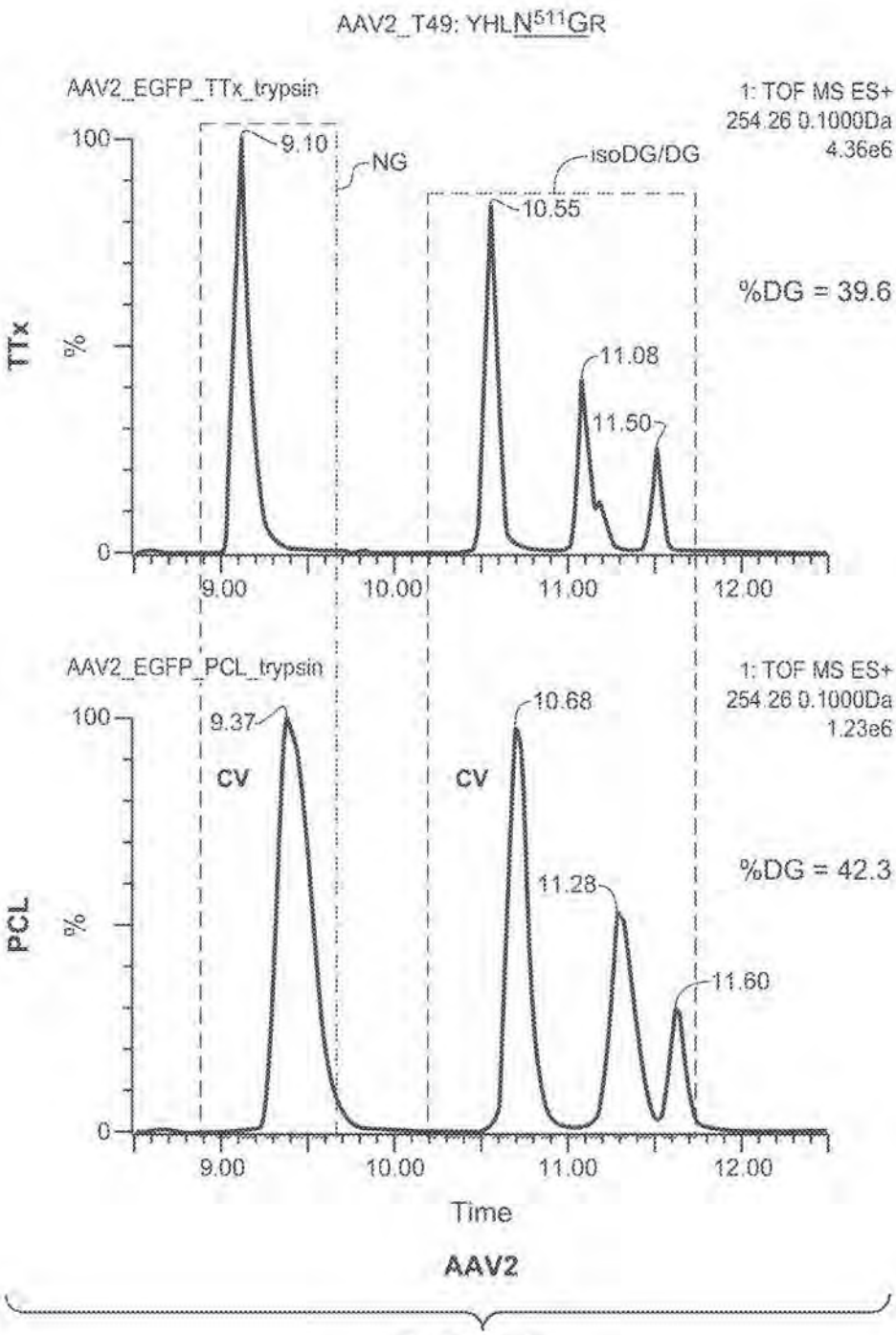

AAV2_T49: YHLN511GR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
254.26 0.1000Da
4.36e6
100
9.10
NG
isoDG/DG
10.55
%DG = 39.6
11.08
11.50
TTx
%
0
9.00
10.00
11.00
12.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
254.26 0.1000Da
1.23e6
100
9.37
CV
CV
10.68
%DG = 42.3
11.28
11.60
PCL
%
0
9.00
10.00
11.00
12.00
Time
AAV2

FIG. 7B

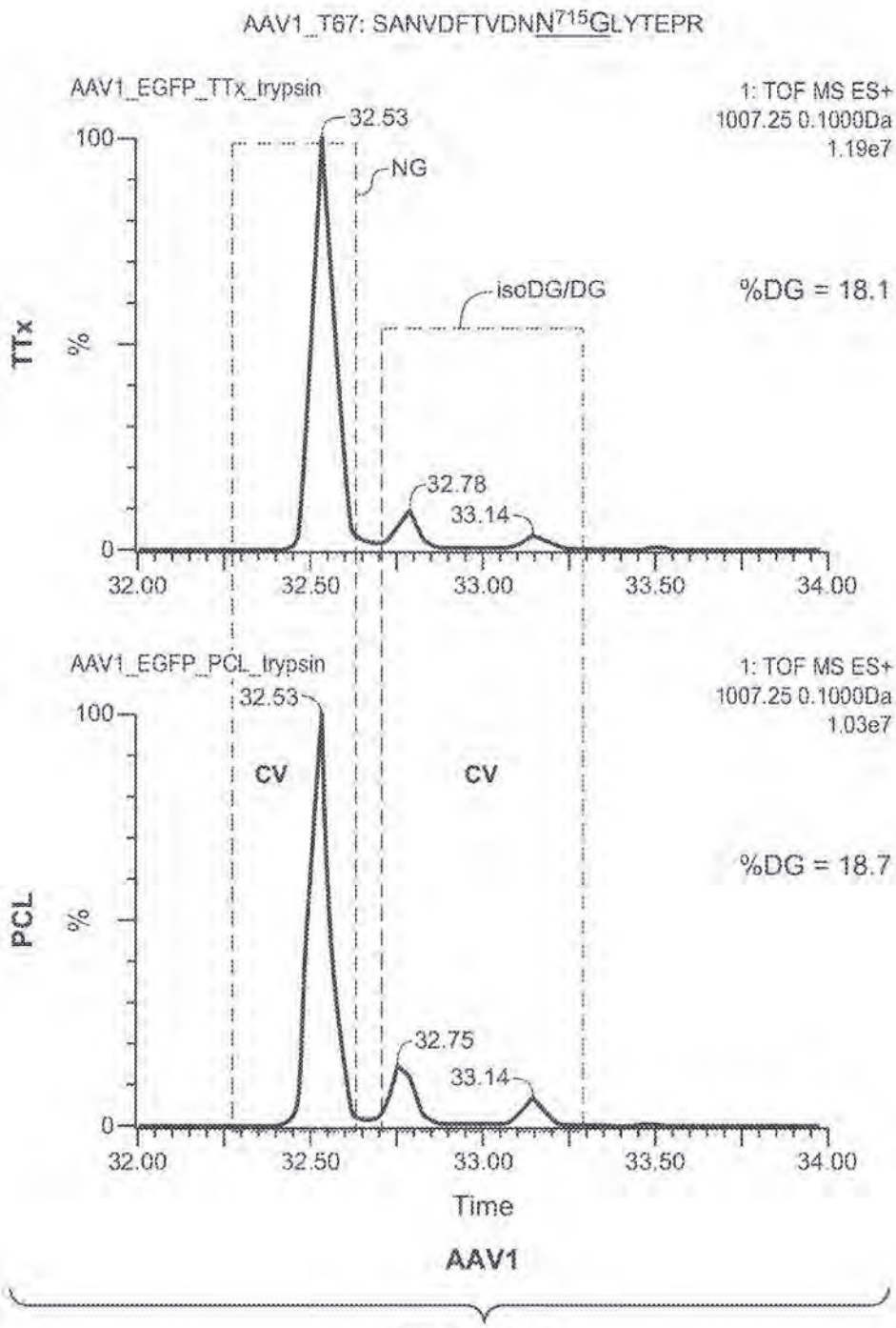

AAV1_T67: SANVDFTVDNN715GLYTEPR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.19e7
32.53
NG
isoDG/DG
%DG = 18.1
TTx
%
100
0
32.78
33.14
32.00
32.50
33.00
33.50
34.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.03e7
32.53
CV
CV
%DG = 18.7
PCL
%
100
0
32.75
33.14
32.00
32.50
33.00
33.50
34.00
Time
AAV1

FIG. 8A

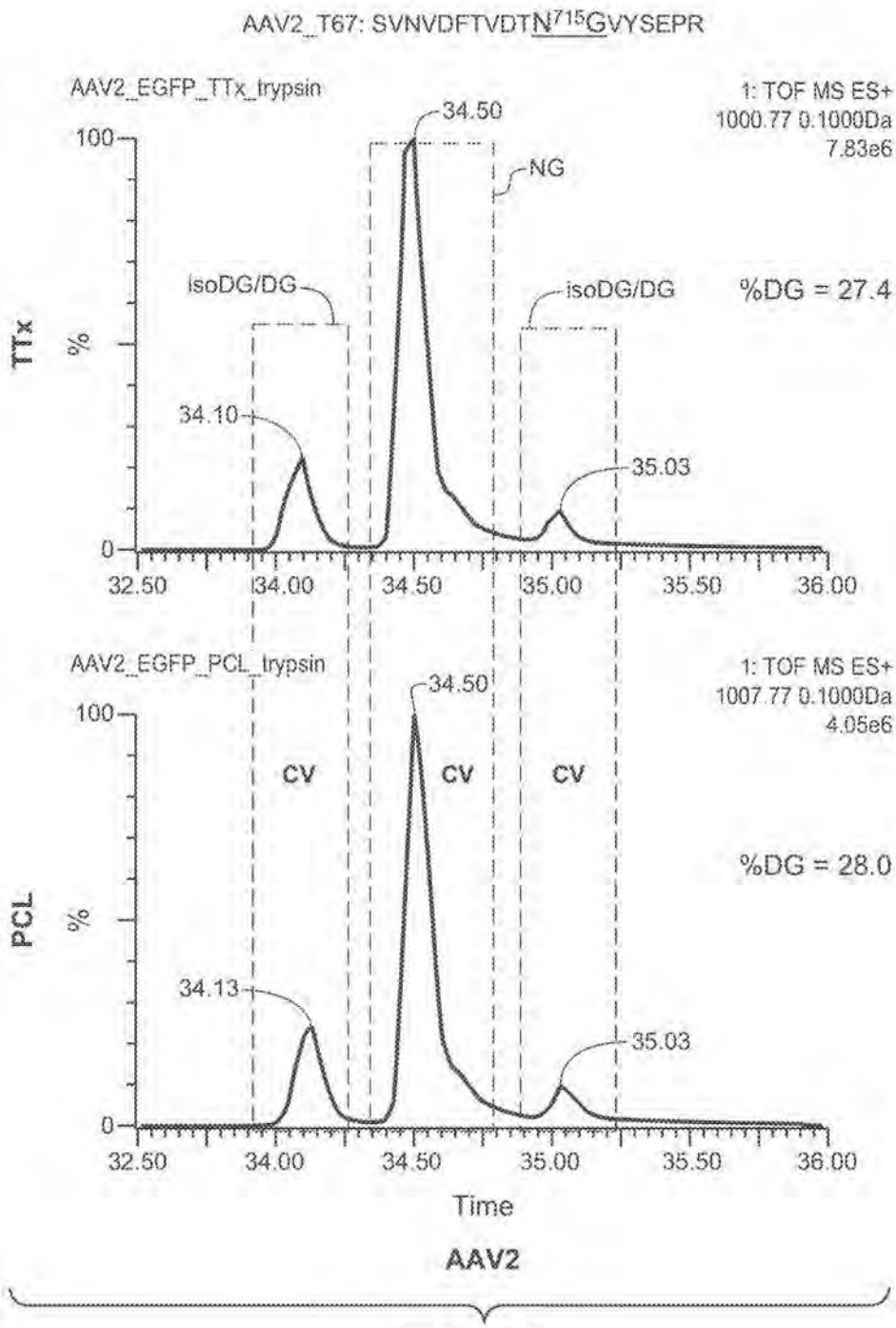
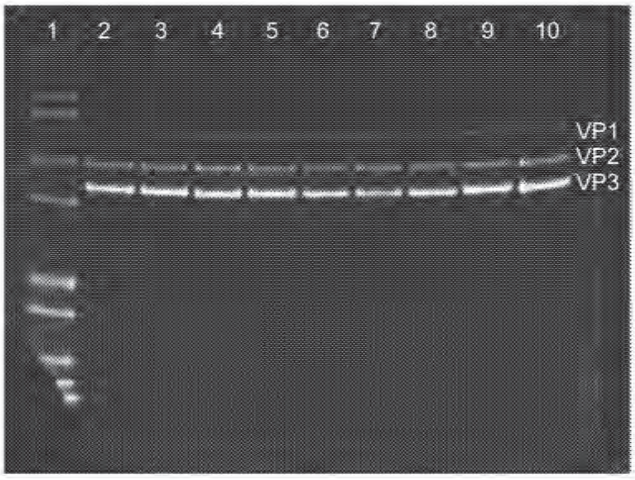

AAV2_T67: SVNVDFTVDTN715GVYSEPR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
1000.77 0.1000Da
7.83e6
34.50
NG
isoDG/DG
isoDG/DG
%DG = 27.4
34.10
35.03
TTx
%
100
0
32.50
34.00
34.50
35.00
35.50
36.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
1007.77 0.1000Da
4.05e6
34.50
CV
CV
CV
%DG = 28.0
34.13
35.03
PCL
%
100
0
32.50
34.00
34.50
35.00
35.50
36.00
Time
AAV2

FIG. 8B



Deacetylation AAV5 Mutant Vectors

Lane	Sample
1	Mark12 Marker
2	deAC-AAV5 (S2G)/CBA-eGFP X17052C
3	deAC-AAV5 (S2L)/CBA-eGFP X17052D
4	deAC-AAV5 (S2GVP3)/CBA-eGFP X17058D
5	deAC-AAV5 (S2LVP3)/CBA-eGFP X17065A
6	deAC-AAV5 (S2PVP1VP3)/CBA-eGFP X17065B
7	deAC-AAV5 (S2GVP1VP3)/CBA-eGFP X17065C
8	deAC-AAV5 (S2PVP3)/CBA-eGFP X17065D
9	deAC-AAV5 (S2P)/CBA-eGFP X17065E
10	deAC-AAV5 (S2LVP1VP3)/CBA-eGFP X17072B

(~1E10 VGs/Lane)

FIG. 9

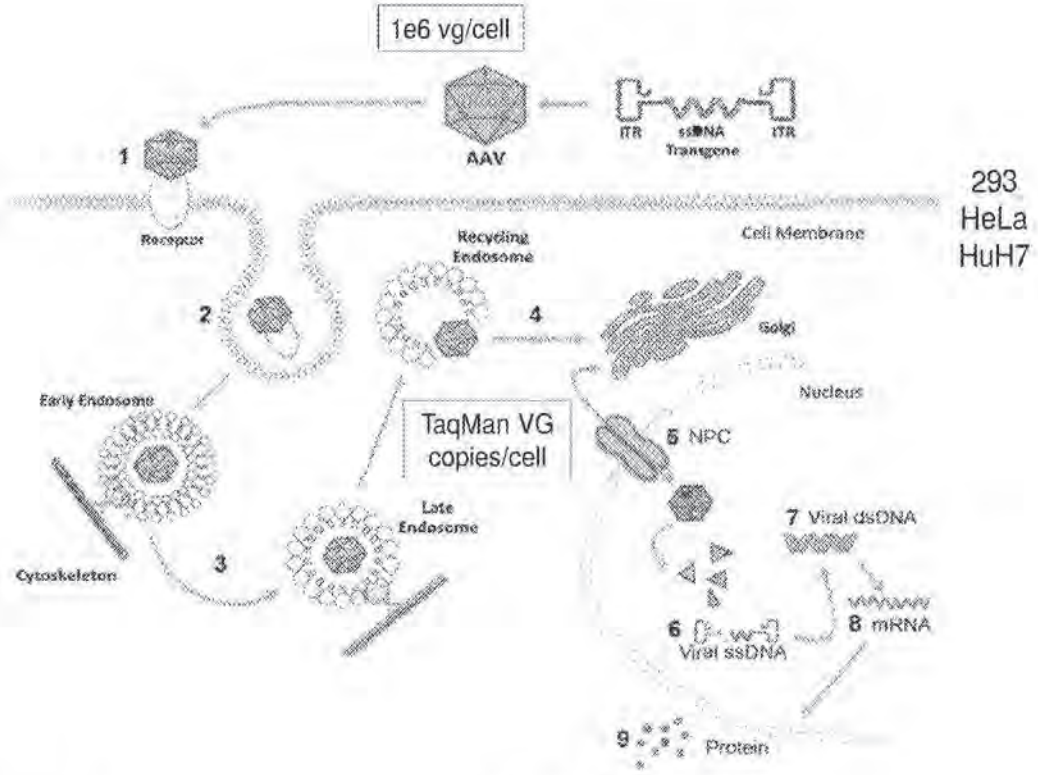


FIG. 10

eGFP ELISA

FIG. 11

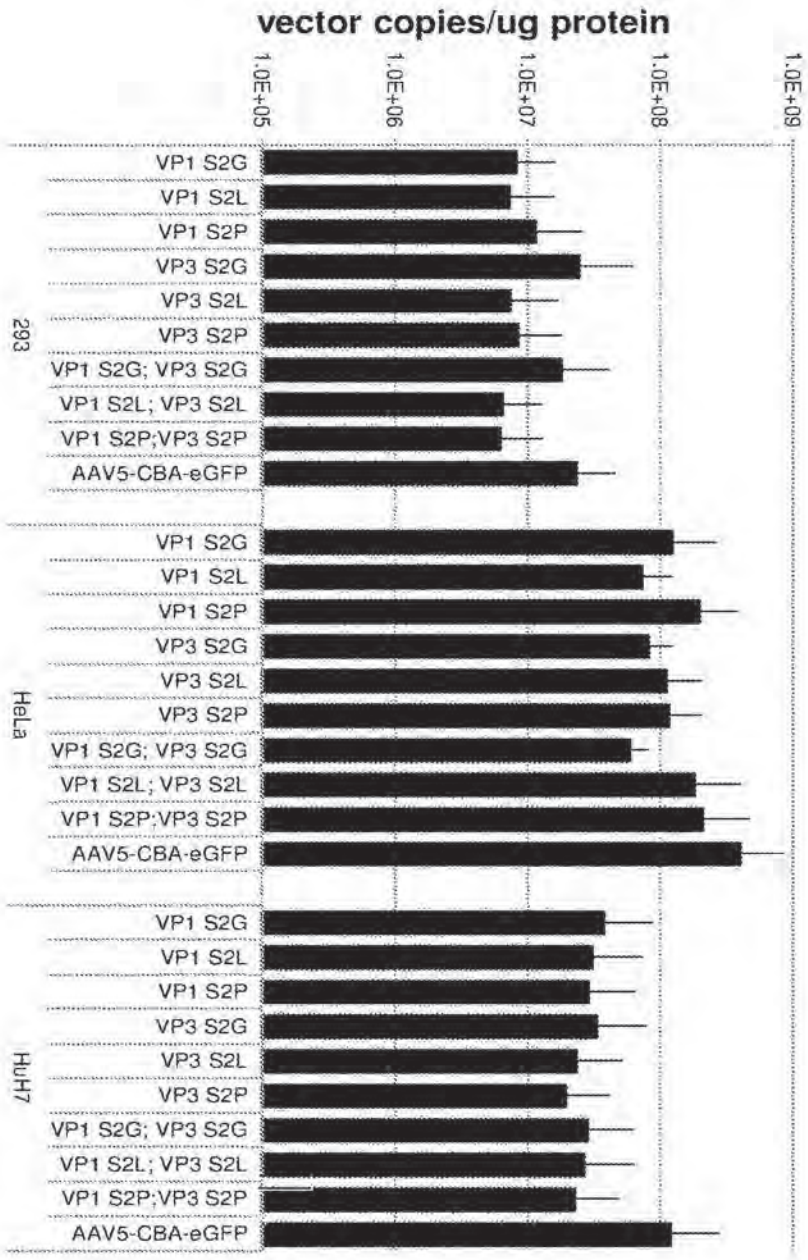
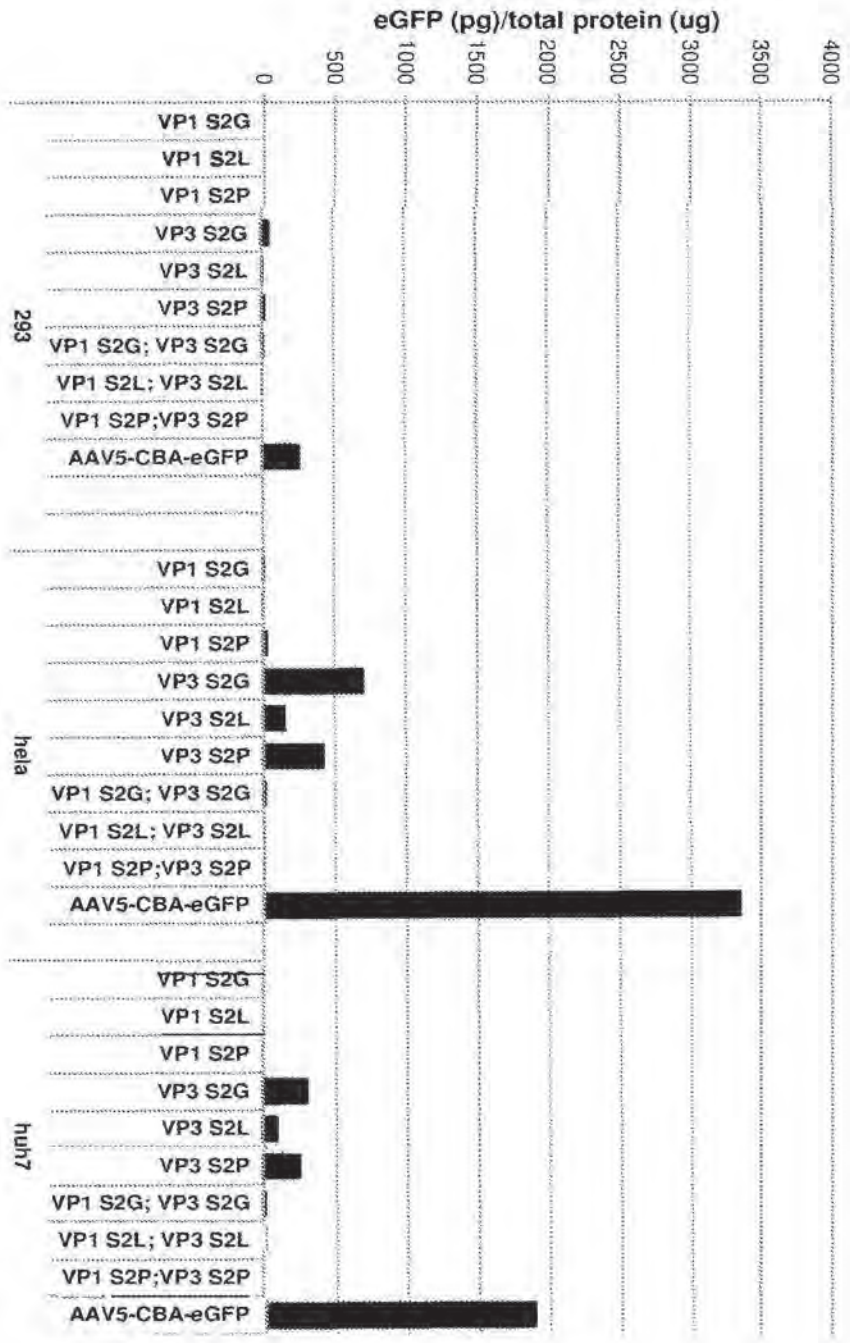

vector copies/ug protein
1.0E+05
1.0E+06
1.0E+07
1.0E+08
1.0E+09
293
HeLa
HuH7
VP1 S2G
VP1 S2L
VP1 S2P
VP3 S2G
VP3 S2L
VP3 S2P
VP1 S2G; VP3 S2G
VP1 S2L; VP3 S2L
VP1 S2P;VP3 S2P
AAV5-CBA-eGFP

FIG. 12


eGFP (pg)/total protein (ug)
0
500
1000
1500
2000
2500
3000
3500
4000
293
VP1 S2G
VP1 S2L
VP1 S2P
VP3 S2G
VP3 S2L
VP3 S2P
VP1 S2G; VP3 S2G
VP1 S2L; VP3 S2L
VP1 S2P;VP3 S2P
AAV5-CBA-eGFP
hela
VP1 S2G
VP1 S2L
VP1 S2P
VP3 S2G
VP3 S2L
VP3 S2P
VP1 S2G; VP3 S2G
VP1 S2L; VP3 S2L
VP1 S2P;VP3 S2P
AAV5-CBA-eGFP
huh7
VP1 S2G
VP1 S2L
VP1 S2P
VP3 S2G
VP3 S2L
VP3 S2P
VP1 S2G; VP3 S2G
VP1 S2L; VP3 S2L
VP1 S2P;VP3 S2P
AAV5-CBA-eGFP

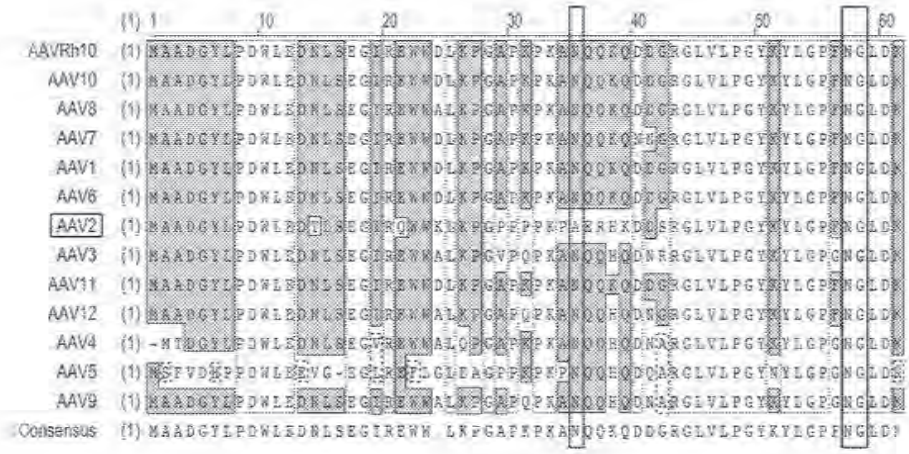

AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 13

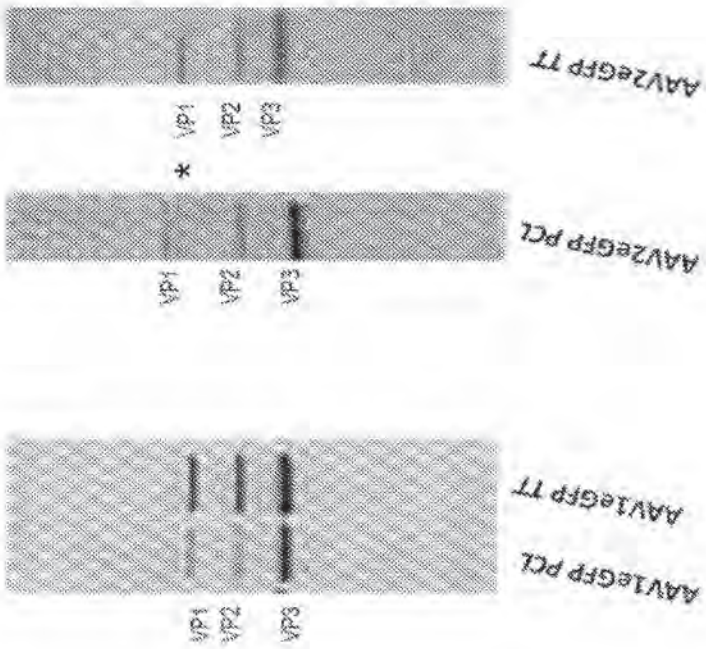
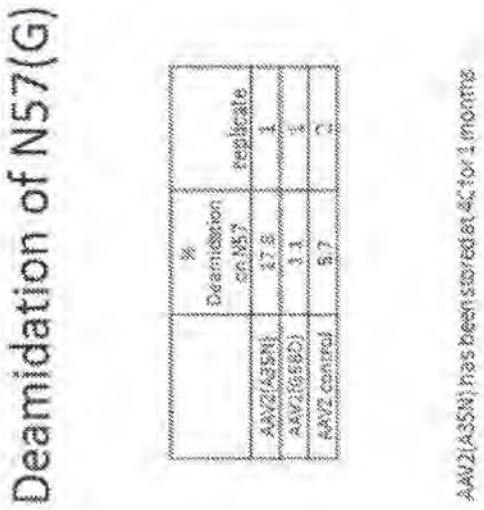
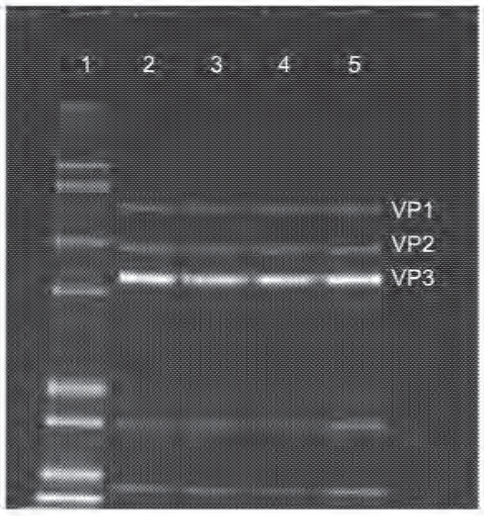

AAV2eGFP TT
VP1
VP2
VP3
*
AAV2eGFP PCL
VP1
VP2
VP3
AAV1eGFP TT
AAV1eGFP PCL
VP1
VP2
VP3

FIG. 14



	% Deamidation on N57	replicate
AAV2(A435N)	17.8	1
AAV2(T265D)	3.1	1
AAV2 control	5.7	2

FIG. 15



Deamidation AAV2 Mutant Vectors

Lane	Sample
1	Mark12 Marker
2	deAMID-AAV2 (N57K)/CBA-eGFP X17079B
3	deAMID-AAV2 (N57D)/CBA-eGFP X17079C
4	deAMID-AAV2 (N57Q)/CBA-eGFP X17079D
5	deAMID-AAV2 (A35N)/CBA-eGFP X17079E

(~5E10 VGs/Lane)

FIG. 16

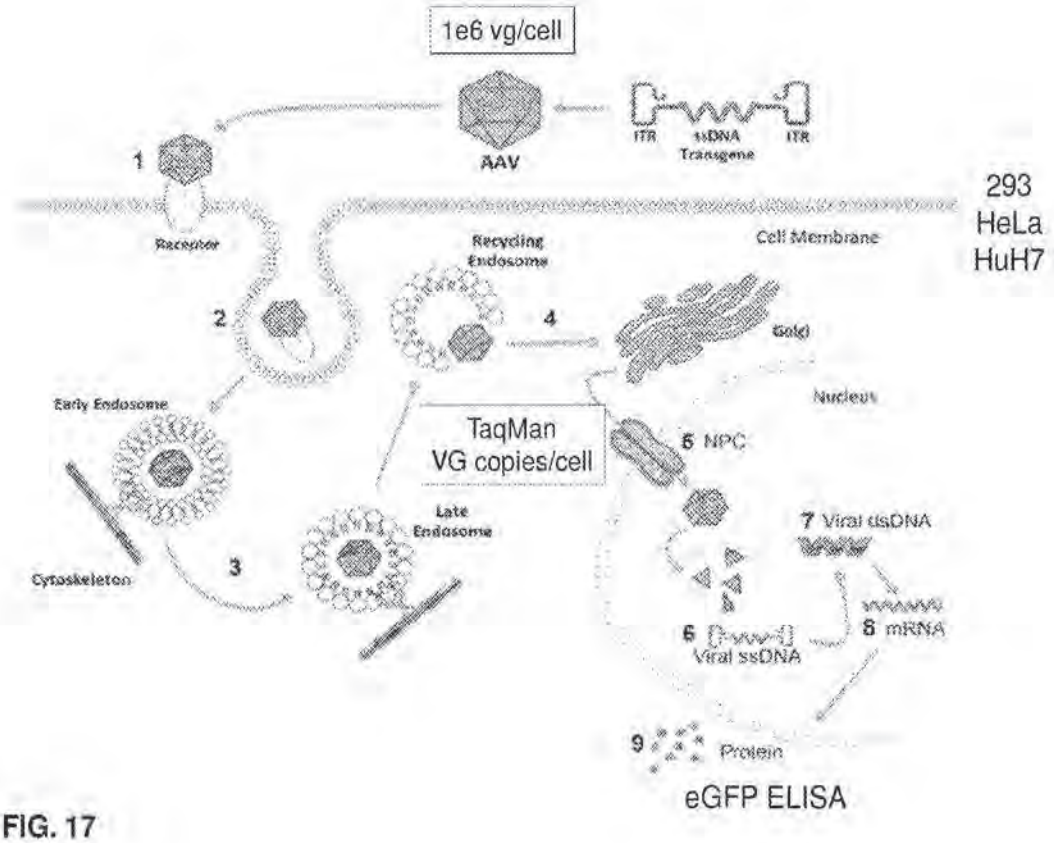

1e6 vg/cell
AAV
ITR
ssDNA
Transgene
ITR
1
Receptor
293
HeLa
HuH7
Recycling
Endosome
Cell Membrane
2
4
Golgi
Early Endosome
Nucleus
TaqMan
VG copies/cell
5 NPC
Late
Endosome
7 Viral dsDNA
Cytoskeleton
3
6
Viral ssDNA
8 mRNA
9
Protein
eGFP ELISA

FIG. 17

FIG. 18

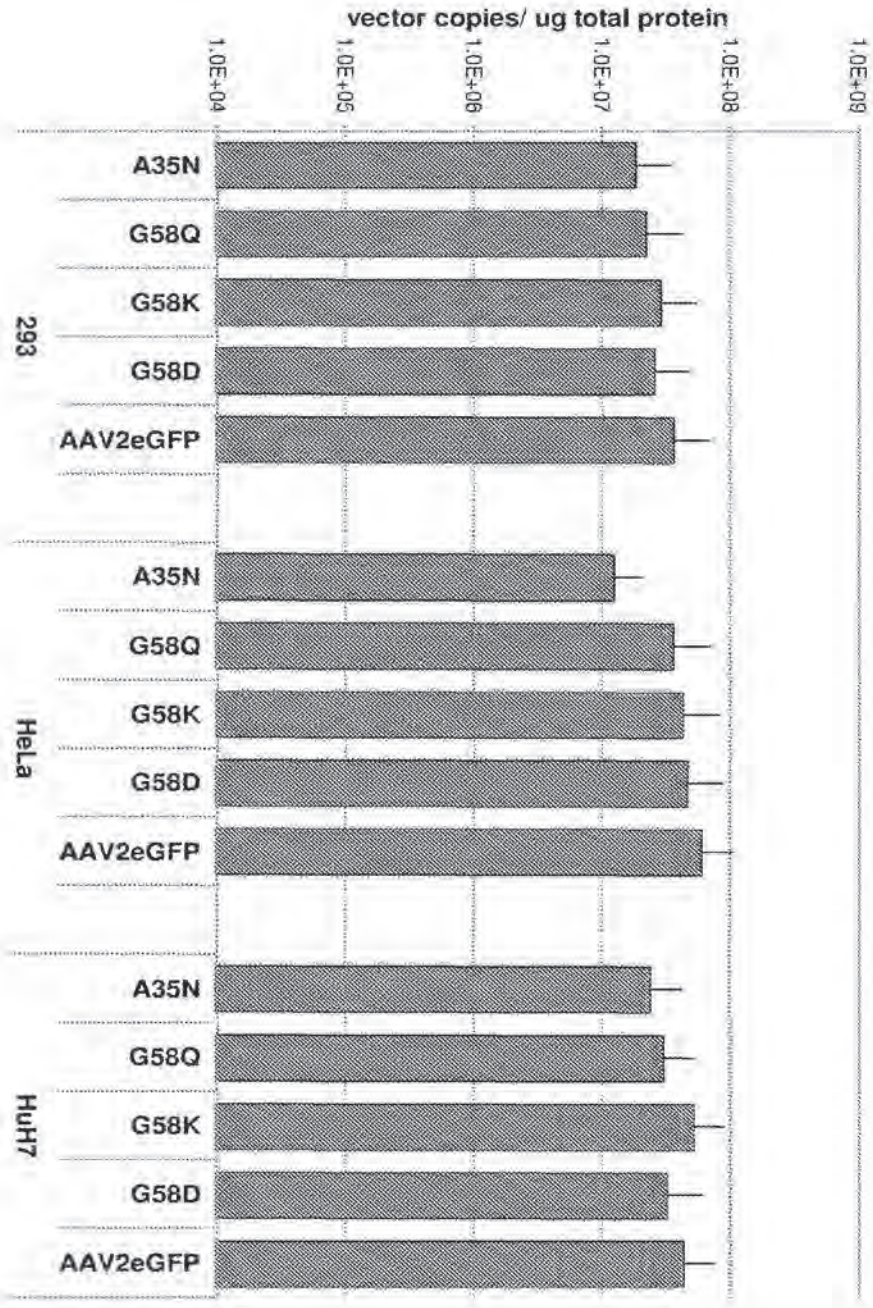
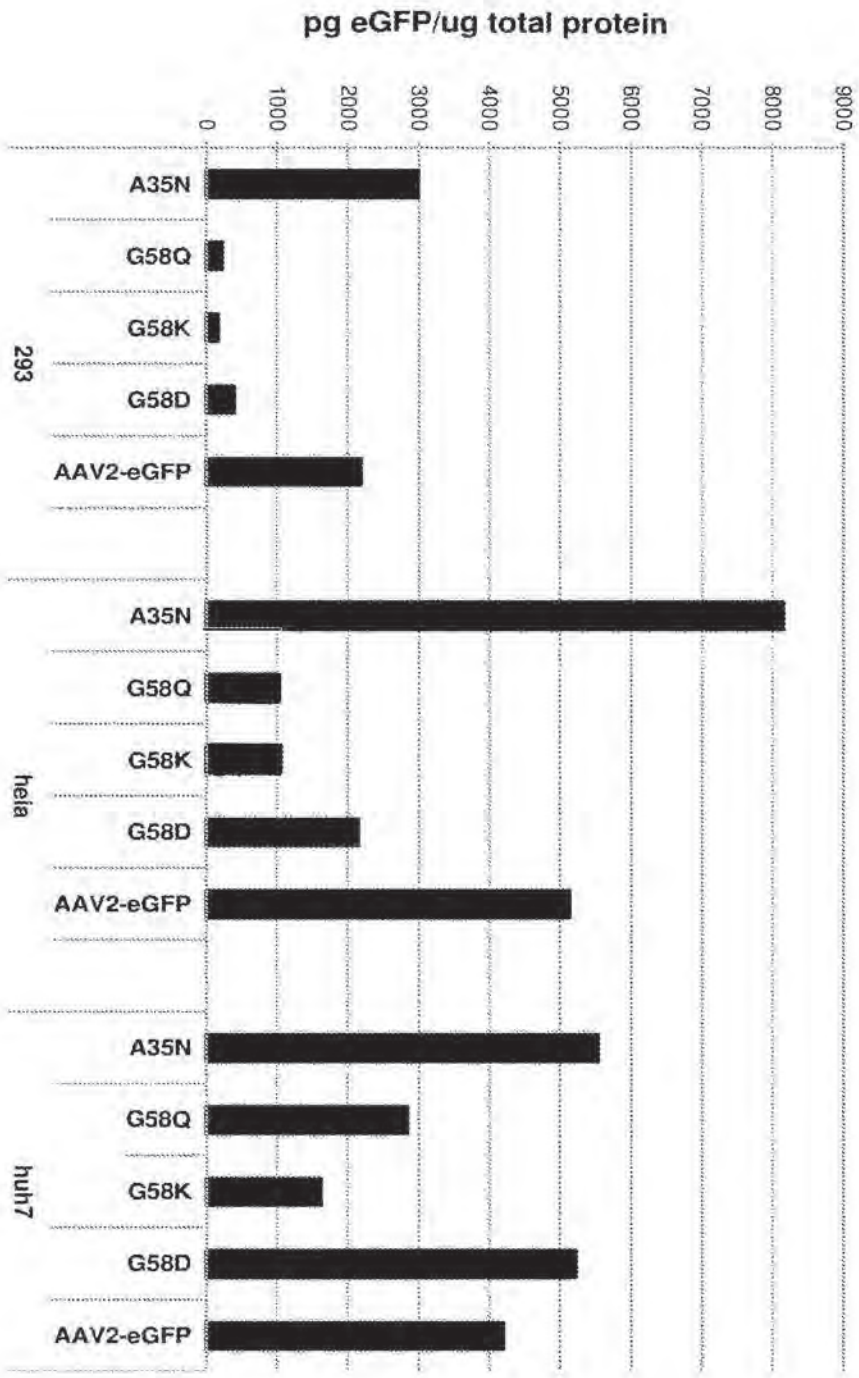

vector copies/ ug total protein
1.0E+04
1.0E+05
1.0E+06
1.0E+07
1.0E+08
1.0E+09
293
A35N
G58Q
G58K
G58D
AAV2eGFP
HeLa
A35N
G58Q
G58K
G58D
AAV2eGFP
HuH7
A35N
G58Q
G58K
G58D
AAV2eGFP

FIG. 19


pg eGFP/ug total protein
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
293
A35N
G58Q
G58K
G58D
AAV2-eGFP
hela
A35N
G58Q
G58K
G58D
AAV2-eGFP
huh7
A35N
G58Q
G58K
G58D
AAV2-eGFP