Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

Analytical Ultracentrifugation as an Approach to Characterize Recombinant Adeno-Associated Viral Vectors

Brenda Burnham, Shelley Nass, Elton Kong, MaryEllen Mattingly, Denise Woodcock, Antonius Song, Samuel Wadsworth,[†] Seng H. Cheng, Abraham Scaria, and Catherine R. O'Riordan*

Gene Therapy, Genzyme, a Sanofi Company, Framingham, Massachusetts.
[†]Current address: Dimension Therapeutics, Cambridge, Massachusetts.
This article was originally presented as an Abstract at the ASGCT meeting, May 13–16, 2015, New Orleans.

Recombinant adeno-associated viral (rAAV) vectors represent a novel class of biopharmaceutical drugs. The production of clinical-grade rAAV vectors for gene therapy would benefit from analytical methods that are able to monitor drug product quality with regard to homogeneity, purity, and manufacturing consistency. Here, we demonstrate the novel application of analytical ultracentrifugation (AUC) to characterize the homogeneity of preparations of rAAV vectors. We show that a single sedimentation velocity run of rAAV vectors detected and quantified a number of different viral species, such as vectors harboring an intact genome, lacking a vector genome (empty particles), and containing fragmented or incomplete vector genomes. This information is obtained by direct boundary modeling of the AUC data generated from refractometric or UV detection systems using the computer program SEDFIT. Using AUC, we show that multiple parameters contributed to vector quality, including the AAV genome form (i.e., self-complementary vs. single-stranded), vector genome size, and the production and purification methods. Hence, AUC is a critical tool for identifying optimal production and purification processes and for monitoring the physical attributes of rAAV vectors to ensure their quality.

INTRODUCTION

GENE TRANSFER VECTORS based on adeno-associated virus (AAV) have demonstrated significant promise for human gene therapy. Ongoing clinical trials for the blinding disorder termed Leber's congenital amaurosis 2[1–8] and the blood clotting disorder hemophilia B[9] have demonstrated that AAV-based gene therapies can be safe and efficacious. Moreover, with the recent approval in Europe of a recombinant AAV1 serotype vector for the treatment of a lipoprotein lipase deficiency, recombinant adeno-associated viral (rAAV) vectors are considered to be a novel class of biological drugs.[10] To support the manufacturing of clinical-grade rAAV therapeutics, reliable, scalable, and GMP-compliant production and characterization protocols are needed. Although significant improvements for rAAV vector production and purification have been reported,[11] methods to monitor the physical attributes of clinical-grade rAAV vectors to ensure their quality are lacking. For example, no established approaches exist for assessing the homogeneity of rAAV vector preparations that include measuring both the presence and fractional content of empty viral particles, full genome-containing viral vectors, and vectors with fragmented genomes or non-transgene-related DNA contaminants. Thus, identifying and developing sensitive and accurate methods to assess vector quality are needed. Although analytical ultracentrifugation (AUC) has been applied to the characterization of recombinant adenovirus vectors,[12,13] its application in the characterization of rAAV vectors to assess particle heterogeneity and quality has not been described. The recent advances in AUC methodology, in particular, modern computational strategies, have allowed for the direct modeling of the sedimentation process of heterogeneous mixtures, generating sedimentation velocity size distribution

*Correspondence: Dr. Catherine R. O'Riordan, Gene Therapy, Genzyme, a Sanofi Company, 49 New York Avenue, Framingham, MA 01701-9322. E-mail: catherine.oriordan@genzyme.com

© 2015 by Mary Ann Liebert, Inc.
DOI: 10.1089/hgtb.2015.048

Genzyme Ex. 2019
Sarepta Therapeutics, Inc. v. Genzyme Corporation
Page 1 of 15

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

analyses with improved detection limits and enhanced resolution.[14] Additionally, the introduction of mathematical algorithms,[15] to allow the solution of the differential equation of the sedimentation/diffusion process, allows a quantitative assessment of constituent particles of a heterogeneous mixture. Consequently, the use of AUC has expanded to include analysis of protein–protein interactions,[16] solutions of polydisperse nanoparticles,[17] viruses,[12,13] and therapeutic viral vaccines.[18]

AAVs harbor attractive features for gene transfer. Wild-type AAV is composed of a single-stranded DNA genome that encodes replication (rep) and encapsidation (cap) genes flanked by inverted terminal repeats (ITRs).[19,20] Recombinant AAV-based vectors are typically produced by a transient transfection method using the "gutless" vector approach.[21–23] The therapeutic genes of interest, together with the regulatory elements, are inserted between the 5′ and 3′ viral ITRs and are encoded on one plasmid. The rep and cap functions are provided in trans on a separate plasmid, and a third plasmid provides the required adenoviral helper genes, E4, E2, and Va RNA. The transfection of the three plasmids is postulated to result in viral vectors that are fully assembled, and the ITR-flanked vector genome is then inserted into the virus via a capsid pore,[24] the resulting population of viral vectors contains both non-genome-containing particles (empty capsids) and genome-containing vectors. In addition, the preparation may contain incomplete portions of the rAAV genome or nongenomic nucleic acid contaminants. As adaptation of the transient transfection protocol to a manufacturing process is challenging, alternate scalable production methods such as those using baculovirus,[25–28] herpes simplex HSV,[29,30] or packaging/producer cell lines[31–38] are being considered. However, the production method likely influences the quality of the final rAAV vector product, particularly with respect to the amounts of empty particles, capsids containing fragmented genomes, and capsids containing nongenomic nucleic acid contaminants. This idea is significant, particularly in light of recent reports that rAAV vectors that are fully or partially empty exacerbate vector-related side effects and negatively affect gene transduction.[39] Thus, an analytical method to quantitate the fractional content of these various vector species is desirable for clinical vector lot release.

AAV vectors package single-stranded genomes; therefore, they require host-cell synthesis of the complementary strand for efficient transduction. The use of dimeric or self-complementary molecules (scAAV) eliminates the requirement for host-cell DNA synthesis.[40] scAAV vectors fold upon themselves to form transcriptionally competent double-stranded DNA. AAV vector genome dimerization is achieved by mutation or deletion of one of the two terminal resolution sites (trs).[40] A recent clinical trial for hemophilia B, using AAV8*FIX*,[9] demonstrated the clinical potential of self-complementary AAV vectors, but the generation and characterization of these vectors for clinical use remain a challenge. The fractional content of the capsids with dimeric genomes needs to be accurately quantitated and shown to be reproducible between different clinical vector lots. For the successful manufacturing of scAAVs, factors that result in the generation and packaging of monomeric genomes need to be identified to minimize the fractional content of vectors that harbor "half size" or monomeric genomes.

Early studies using preparative ultracentrifugation demonstrated that wild-type AAV2 virus consists of a range of particles of different buoyant densities.[41] Using a combination of cesium chloride (CsCl) density centrifugation and velocity sedimentation in sucrose gradients, De La Maza and Carter[41] demonstrated that wild-type AAV2 virus is composed of virus particles containing genomes of varying size and sedimentation coefficients. Consequently, preparative ultracentrifugation in CsCl density gradients is routinely used to fractionate rAAV vector preparations into their constitutive virus particles.[39] However, the quantitative analysis of the various rAAV particles following fractionation by preparative ultracentrifugation is challenging as the recovery of distinct virus populations from the cesium gradient is not always achievable. Here, we show that AUC offers many advantages over preparative ultracentrifugation. One advantage is that particle separation can be monitored during the centrifugation *in situ* and in real time; therefore, a quantitative assessment of constituents of rAAV vector preparations can be determined.

AUC demonstrated that rAAV vector preparations contained different species of rAAV with distinct densities, which include viral particles containing a full rAAV genome, particles that are totally empty or partially empty as a result of packaging fragmented vector genomes, or other non-vector genomic nucleic acid contaminants. In addition, AUC demonstrated that different production and purification processes and vector genome size and form (single-stranded vs. self-complementary) all contribute to rAAV vector heterogeneity. Thus, AUC provides a critical tool to identify and quantitate all virion particle species in rAAV vector preparations. In addition, AUC can be

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

used to evaluate the strategies that help minimize the fractional content of nonfunctional empty or partially empty virions.

MATERIALS AND METHODS

Vector production and purification

AAV vectors were produced either via transient transfection,[23] or via the producer cell method,[35,37] as previously described. For the production of AAV vectors by transfection, HEK293 cells were transfected using polyethyleneimine, PEI, and a 1:1:1 ratio of the three plasmids (ITR vector, AAV rep/cap, and Ad helper plasmid). In the case of self-complementary AAV vectors, the ITR vector plasmid contained one wild-type and one mutated ITR as described.[40] The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, CA). For the generation of the empty capsids, the vector ITR plasmid was omitted, and only empty capsids were assembled. Generation of AAV vectors by the producer cell line method was performed as described previously.[35,37] Briefly, a HeLa-based producer cell line was created following transfection of HeLaS3 cells (ATCC CCL-2.2) with a single plasmid containing the following elements: AAV2 rep genes and cap gene of the desired serotype, the vector genome flanked by AAV2 ITRs, and a puromycin resistance gene. Transfected cells were grown in the presence of puromycin to isolate stable integrants, which were subsequently screened for AAV productivity following infection with wtAd5 virus.[35,37] Purification of AAV from both production platforms was performed as previously described.[42] A description of all AAV vectors used for analysis in this study, with their method of production, is included as Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/hgtb).

Sample preparation for AUC analysis

Purified vector, at a concentration of 2e12–5e12 vg/ml, was buffer exchanged into phosphate-buffered saline (PBS), pH 7.2, using a 10K MWCO Slide-a-Lyzer (Thermo Scientific, Waltham, MA). The AAV vector absorbance signal was determined by optical density measurement at 260 nm (OD_{260}) using spectrophotometric methods. For consistency, the samples were adjusted to a target concentration (OD_{260} of between 0.2 and 0.8) by direct dilution with PBS or further concentrated using an Amicon Ultra-0.5/30K MWCO Centrifugal Filter Device (Merck, KGaA, Darmstadt, Germany). AAV empty particle concentration was determined by AAV Titration ELISA (Progen; Biotechnik, Heidelberg, Germany) or A_{260}/A_{280}.

Sedimentation velocity AUC data acquisition

Sedimentation velocity AUC analysis was performed using a Proteome Lab XL-I (Beckman Coulter, Indianapolis, IN). Four hundred microliters of sample was loaded into the sample sector of a two-sector velocity cell, and 400 μl of 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 a full vacuum were maintained for 1 hr. Sedimentation velocity centrifugation was performed at 20,000 rpm and 20°C. Absorbance (260 nm) and Raleigh interference optics were used to simultaneously record the radial concentration as a function of time until the lightest sedimenting component cleared the optical window (1.2 hr).

AUC data analysis

The percentage of virions containing a full genome was determined by analyzing approximately 60 scans using either the absorbance or interference detection method and the SEDFIT continuous-size C(S) distribution model.[15] Second-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 fits the data to the Lamm equation, and the resulting size distribution is a "distribution of sedimentation coefficients" that resembles a chromatogram with the area under each peak proportional to the 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. The results of the AUC analyses are plotted as the normalized differential coefficient distribution value, C(S), versus sedimentation coefficient (S). Each AUC run is an independent assay, and each analysis is monitored for the following attributes to ensure result quality: goodness of fit (rmsd), the ratio of OD_{260} nm/interference signal in fringes (A260/IF ratio) for each peak, sedimentation coefficient consistency for each species between runs, and the overall scan quality.

Absorbance optics (260 nm)

Absorbance data require the use of extinction coefficients to calculate the molar concentration and the percent value of the empty and genome-containing capsids. Molar concentrations of both

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

genome-containing and empty capsid were calculated using Beer's law and % full genome-containing and empty capsid was calculated. The molar extinction coefficient for AAV DNA containing capsid was determined using the following formula $\varepsilon_{260/\mathrm{DNA}} + \varepsilon_{260/\mathrm{AAV\ capsid}}$.[43]

$$\varepsilon_{260/\mathrm{DNA\ containing\ capsid}} = (20.0 \times \mathrm{MW_{DNA}} + 3.72\mathrm{e}6\ M^{-1}\mathrm{cm}^{-1}) \times \mathrm{cp/vg} = M^{-1}\mathrm{cm}^{-1},$$

where cp/vg = 1.[44] The number of nucleotides is used to calculate the molecular weight of the packaged DNA. The molecular weight of the bases are as follows: A is 312.2 Da, C is 288.2 Da, G is 328.2 Da, and T is 303.2 Da, with the average value of 1 nt = 309.5 g/mol.[43]

The molar extinction coefficient for $\varepsilon_{260/\mathrm{AAV\ capsid}} = 3.72\mathrm{e}6\ M^{-1}\mathrm{cm}^{-1}$.[43]

The molar concentration (M) of each species was calculated using Beer's law.

$$\mathrm{M_{peakX}} = \mathrm{Absorbance_{peakX}} / (\varepsilon_{260\mathrm{peakX}} \times 1.2\mathrm{cm})$$

The relative percentage of each peak in the C(S) distribution is calculated based on the molar concentration of each species in relation to the sum of the molar concentration of all species in the distribution.

An example with three peaks in the distribution is as follows:

$$\%_{\mathrm{peak\ 1}} = [\mathrm{M_{peak1}} / (\mathrm{M_{peak1}} + \mathrm{M_{peak2}} + \mathrm{M_{peak3}})] \times 100$$

The C(S) values were determined using the SEDFIT algorithm described.[15] The molar concentration of both the genome-containing vector and the empty virion was calculated using Beer's law, and the fractional content of each capsid species was reported as a % of the total.

Southern blot analysis

Vector DNA was extracted from approximately 1×10^{11} vector capsids by digestion in 50 μl reactions containing 0.4 mg/ml protease K, 1% sarkosyl, and 10 mM EDTA at 50°C for 1 hr, followed by phenol/chloroform extraction. The samples were then precipitated with ethanol for analysis by alkaline agarose gel electrophoresis and Southern blot hybridization as previously described by McCarty et al.[40]

RESULTS

Optimization of the AUC method for the characterization of rAAV vectors

AUC using the classical boundary sedimentation velocity was used to reveal the particle heterogeneities of the rAAV vector preparations. A mixture of rAAV2 vectors (20% containing a full genome and 80% empty particles) was generated by mixing together defined ratios of purified empty and full genome-containing vectors. To monitor the movement of rAAV2 particles in response to a centrifugal force, this mixture of empty and genome-containing rAAV2 capsids was scanned at 260 nm along a centrifugal field at defined time intervals. Figure 1A shows a representative scanning profile following centrifugation of the rAAV2 mixture at 20,000 rpm for 1.2 hr (when all the sedimenting species clear the optical window). The scans represent the acquisition of concentration data as a function of radius r, at time t, to yield a series of concentration scans that reveal the complete migration pattern of constituent vector particles in the rAAV2 vector preparation. In these sigmoidal-like curves or boundaries, the leading edge of the curve represents the faster sedimenting species (i.e., rAAV2 vectors containing the full genome), whereas the trailing edge of the curve represents the slower sedimenting species (i.e., the "empty" rAAV2 particles) (Fig. 1A). Plotting the differential sedimentation coefficient distribution value [C(S)] versus the sedimentation coefficient in Svedberg units (S) yields the distribution of sedimentation coefficients for the rAAV2 sample (Fig. 1B). The C(S) values were determined using the SEDFIT algorithm.[15] To calculate the molar concentrations and percent values for each viral species from the absorbance data, extinction coefficients were determined. Molar absorbance extinction coefficients for both the empty particles ($\varepsilon_{260/\mathrm{capsid}} = 3.72 \times 10^6$) and genome-containing particles ($\varepsilon_{260/\mathrm{capsid}} = 3 \times 10^7$) were calculated using genome size and published formulas.[43] Molar concentrations of both genome-containing vectors and empty particles were 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. 1B). These results demonstrate that AUC may be used to accurately distinguish and quantify empty particles and genome-containing vectors in a heterogeneous preparation.

For the $\mathrm{rAAV2_{3466}}$ vector preparation shown in Fig. 1A and B, viral vectors containing a full genome are represented by the peak sedimenting at 94S and accounted for 21% of the vector preparation. Empty capsids sedimented with an S value of 64S and accounted for 79% of the vector preparation. These sedimentation values were confirmed by AUC analysis of pure populations of empty (Fig. 1C) or genome-containing capsids (Fig. 1D). The

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

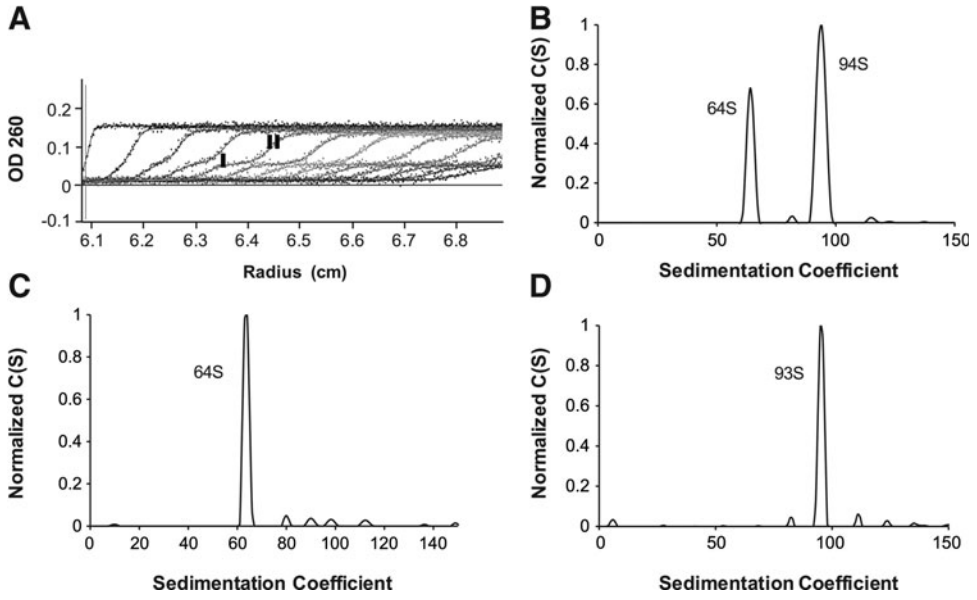


Figure 1. Boundary sedimentation velocity profiles of an AAV2 vector containing a predetermined 4:1 ratio of empty:genome-containing capsids **(A)**. The size of the vector genome is 3465 nucleotides. Two areas are indicated, which correspond to areas associated with the empty capsid (I) and (II) material migrating faster than the empty capsids, that is, the genome-containing capsids. The data shown in **(A)** were then analyzed using SEDFIT to calculate the sedimentation coefficient distribution plot shown in **(B)**. Two distinct populations of virions are represented with the sedimentation coefficients of 64S and 94S, representing empty capsids and genome-containing capsids, respectively. The *X* axis represents the sedimentation coefficient in Svedberg units S, and the *Y* axis represents the concentration as a function of S. The integration of each peak yields the relative concentration of each species in units of detection, and the application of Beer's law ($C = A/\varepsilon l$) yields the concentration of each species. Molar extinction coefficient values for empty capsids and genome-containing capsids were determined according to published formulas[43] and the relative percentage of each species determined. The vector sample contains 79% empty capsids and 21% genome-containing capsids in agreement with the 4:1 predetermined ratio. **(C)** and **(D)** represent the sedimentation coefficient distribution plots for pure populations of empty capsids **(C)** and genome-containing capsids **(D)**, demonstrating that the sedimentation coefficients for empty capsids and genome-containing capsids are 64S and 93S, respectively.

AUC profile of a pure population of AAV2 empty particles revealed a single peak with a sedimentation coefficient of 64S, whereas the AUC profile of a pure population of AAV2 genome-containing vectors revealed a single peak with a higher sedimentation coefficient of 93S. These results support the values generated above and further confirm that AUC may be used to quantify genome-containing and empty rAAV capsids from a heterogeneous preparation containing both species.

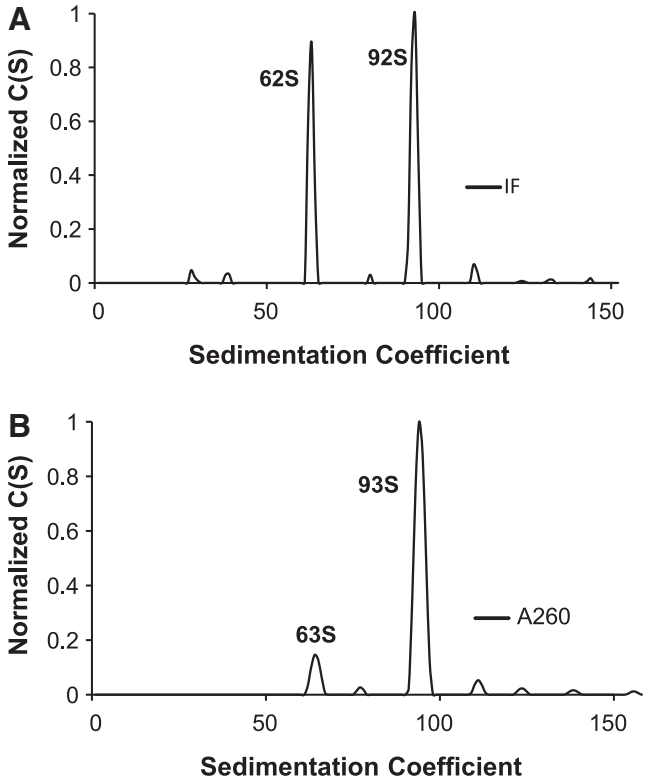
This AUC method was further assessed for reproducibility by performing five independent runs of the same vector sample (an scAAV9 vector harboring a dimeric vector genome of 2050 nucleotides in size, $scAAV9_{2050}$), as shown in Supplementary Table S2. The sedimentation coefficients for both genome-containing and empty rAAV2 vectors were highly reproducible, yielding coefficients of variation from 0.5% to 0.6%. Similarly, the relative abundance (expressed as a percentage of the total) of genome-containing virions was determined with a coefficient of variation of approximately 2%. These results indicate that the AUC method for quantifying genome-containing and empty rAAV2 capsids yields highly reproducible and consistent values.

Comparison of interference and absorbance detection methods

An alternative optical detection method using Rayleigh interference optics (IF) was also evaluated. This detection method measures the sample concentration based on differences in refractive indices between the reference solution and the AAV-containing sample. Similar to absorbance detection, interference detection can be applied to any AAV preparation 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 the concentration.

Pure populations of empty and genome-containing $rAAV2_{3465}$ vectors were mixed at a 1:1 ratio and analyzed by AUC using either interference (Fig. 2A) or absorbance detection methods (Fig. 2B). Interference detection revealed two populations of rAAV particles at the approximate expected ratios of 43%

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.



C

Abs 260 nm		Interference		A260/IF
S	%	S	%	
63	47	62	43	0.41
93	53	92	57	2.38

Figure 2. Comparison of the interference detection and absorbance detection methods. Sedimentation distribution plots for an AAV2 vector containing empty and genome-containing capsids at a ratio of 1:1 using **(A)** interference detection and **(B)** absorbance detection. **(C)** Table comparing the relative percentage of empty and genome-containing capsids as determined by both detection methods. The AAV vector harbored a vector genome-containing 3370 nucleotides. The ratio of absorbance signal to interference signal (A_{260nm}/IF) for both empty and full particles is 0.41 and 2.38, respectively.

empty and 57% genome-containing vectors, as represented by the 62S and 92S species, respectively (Fig. 2A). Both detection methods yielded similar abundance ratios; however, with the absorbance detection method, discordance existed between the peak height and concentration (compare size of 62–63S peaks in Fig. 2A and B). The data generated by both methods are compared in Fig. 2C. The ratio of absorbance signal to interference signal (A_{260nm}/IF) can be used in a manner that is analogous to the 260/280 ratio, which assisted in identifying peaks in the C(S) distribution. Although interference optics offered many advantages over absorbance optics, including greater precision and resolution, this method had the disadvantage of requiring a higher sample concentration. Moreover, the interference optics method was affected by a mismatch between the reference and the rAAV vector sample buffer. The AAV samples typically exhibit a low protein concentration, requiring a more sensitive detection method; also, completely matching the rAAV sample and reference buffers is difficult; therefore, the absorbance optics method was chosen as the preferred optics method for further analysis.

AUC assessment of rAAV vectors produced and purified using different methodologies

The fact that AUC can be a highly accurate and reproducible way to resolve and quantify empty and genome-containing rAAV vectors was applied to analyze the quality of rAAV vector preparations produced using different protocols. A major problem with rAAV vector preparations is the presence of vectors with partial or fragmented genomes. To illustrate the utility of the AUC method to resolve these species, rAAV vectors generated by two different methods, termed the "triple transfection" and "producer cell line" methods, were analyzed by AUC. An $rAAV2_{3370}$ vector, harboring a 3370-nucleotide transgene, was produced using either the triple transfection method or the producer cell line method. AUC analysis showed that the vector preparation profiles produced by these methods were remarkably different. Using the producer cell line method, 74% of the virions contained a full genome, as represented by the 92S species (Fig. 3A), and 19% were empty particles (63S species), with the remainder containing a fragmented genome represented by an intermediary 75S species. By contrast, 82% of the virions produced by the triple transfection method were empty particles (64S species), with 11% containing a fragmented genome (76S and 84S species) and only 8% of the vectors possessing a full genome (94S) (Fig. 3B). Repeat productions of the same $AAV2_{3370}$ vector using either producer cell line or triple transfection yielded similar AUC profiles (Fig. 3C and D). A small variation in the sedimentation coefficient (~2S) between different preparations of the same vector can be explained by small differences in process residuals from the different production runs. In addition, a trend for improved vector quality was evident when the producer cell line method was compared with triple transfection for the production of an AAV2*EGFP* vector harboring

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

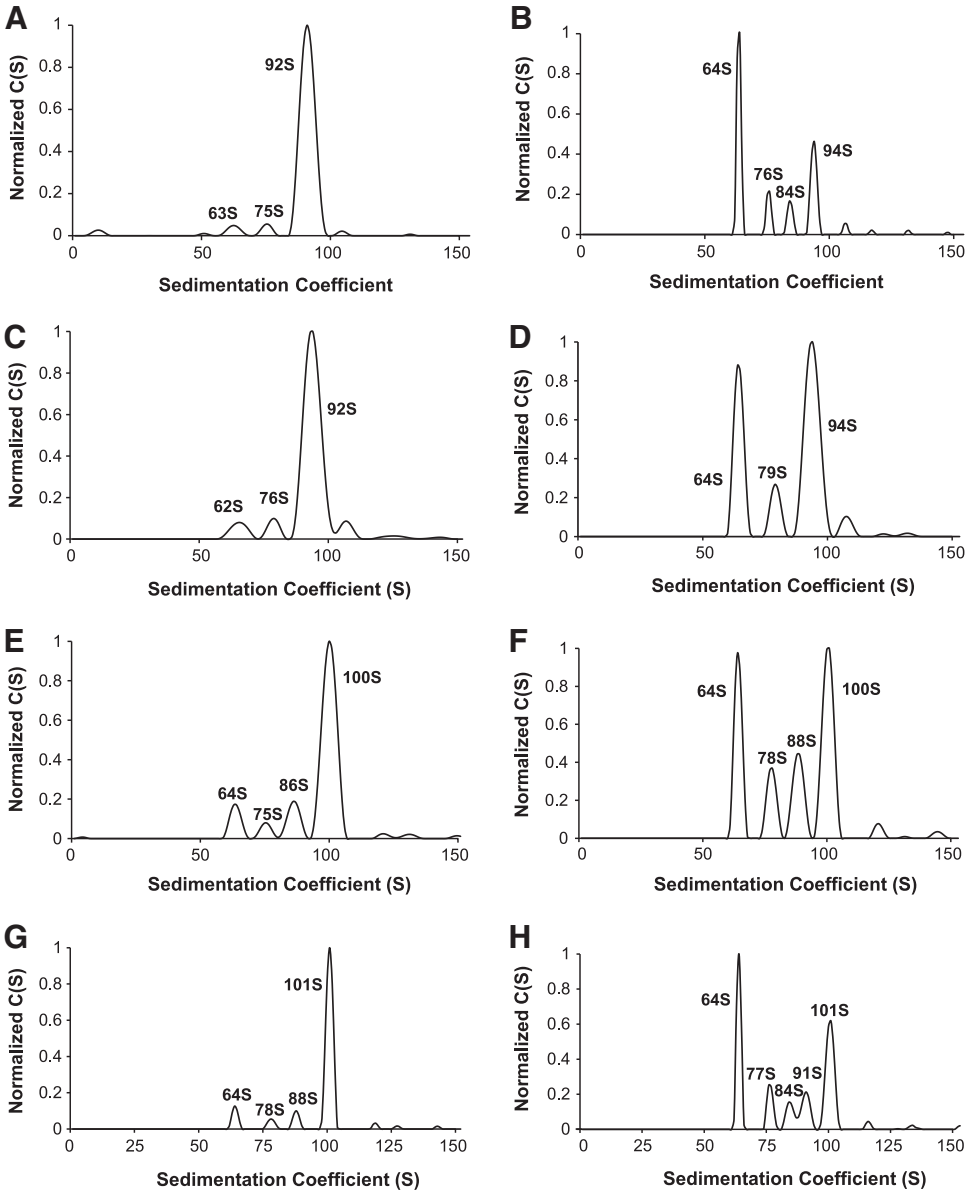


Figure 3. Sedimentation distribution plots for $AAV2_{3370}$ and AAV2*CBAEGFP* vectors generated by the producer cell line **(A, C, E, G)** or triple transfection method **(B, D, F, H)**. **(A)** The relative percentages of each capsid species in an $AAV2_{3370}$ vector generated using the producer cell line method were 19% (63S), 7% (75S), and 74% (92S). **(B)** The relative percentages of each capsid species for the same $AAV2_{3370}$ vector generated using triple transfection were 82% (64S), 7% (76S), 4% (84S), and 8% (94S). **(C, D)** sedimentation distribution plots for repeat productions of $AAV2_{3370}$ using producer cell line **(C)** and triple transfection **(D)**. **(C)** The relative percentages for each capsid were 23% (62S), 7% (76S), and 70% (92S). **(D)** The relative percentages for each capsid were 75% (64S), 7% (79S), and 18% (94S). Sedimentation distribution plots for AAV2*CBAEGFP* vector generated by producer cell line **(E, G)** and triple transfection **(F, H)**. **(E)** The relative percentages of each capsid species in an AAV2*CBAEGFP* vector generated using producer cell line method were 43% (64S), 40% (100S), 7% (75S), and 10% (86S). **(G)** The relative percentages of each capsid species in a repeat production of the same AAV2*CBAEGFP* vector using producer cell line were 42% (64S), 45% (101S), 7% (78S), and 6% (88S). **(F)** The relative percentages of each capsid species for the same AAV2*CBAEGFP* vector generated using triple transfection were 70% (64S), 12% (100S), 10% (78S), and 8% (88S). **(H)** Repeat production of AAV2CBAEGFP by transient transfection yielded relative percentage values of 74% (64S), 10% (101S), 8% (77S), 4% (84S), and 5% (91S).

a larger vector genome of approximately 4200 nucleotides (Fig. 3E–H). These results indicate that vector preparations generated using the producer cell line technology may be of a higher quality, with a greater proportion of the virions containing a full genome. The vast majority of vectors produced by the triple transfection method were empty, with a lower proportion of capsids having a full-length genome. These results also highlight the ability of the AUC method to resolve vectors with fragmented genomes in addition to full genome-containing and empty vectors. Moreover, these

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

findings illustrate the power of the AUC method to evaluate the quality and homogeneity of vector preparations.

Next, the AUC method was used to evaluate the effectiveness of the chromatographic methods at removing empty particles.[42] The separation of the empty and genome-containing vectors was realized using ion exchange chromatography (Fig. 4A, peaks 1 and II). AUC analysis of the different elution peaks revealed that the genome-containing vectors (Fig. 4A, peak II) represented 94% of the vector preparation upon elution from the column. This fraction yielded a single peak with a sedimentation coefficient of 92S by AUC (Fig. 4B). By contrast, the $rAAV2_{3465}$ vector preparation before the chromatographic step (Fig. 4C) contained a substantial number of empty particles. AUC analysis revealed two peaks with S values of 63S and 94S, with the 63S peak (empty particles) representing 52% of the total vector population. These results show that ion exchange chromatographic methods are highly effective in removing empty particles from rAAV vector preparations. Importantly, these results demonstrate the utility of applying the AUC method to evaluate vector quality upon purification and the use of AUC in evaluating different vector purification protocols or techniques.

AUC assessment of viral genome integrity

As illustrated in Figs. 3 and 4, the rAAV vector preparations may contain vectors packaged with fragmented genomes in addition to the full genome and empty capsids. A major problem in generating homogeneous rAAV vector preparations for clinical or research applications is the presence of capsids with fragmented genomes, which may result in aberrant or absent transgene expression. Indeed, heterogeneity associated with rAAV vector preparations has been reported to result from the packaging of fragmented genomes[32] or AAV-encapsidated DNA impurities.[44,45] Therefore, AUC was evaluated as a tool to quantify the aberrant packaging of fragmented genomes in rAAV vector preparations. Empirically determining the extinction coefficient of the fragmented genomes of unknown size and sequence is difficult; therefore, a relationship between the sedimentation coefficient (S value) and genome size was established. To this end, rAAV vector preparations with encapsidated viral genomes of known size were analyzed by AUC, and their corresponding S values were determined. A standard curve was then generated to correlate the genome size and S value, as shown in Fig. 5A. This finding demonstrated a highly linear relationship

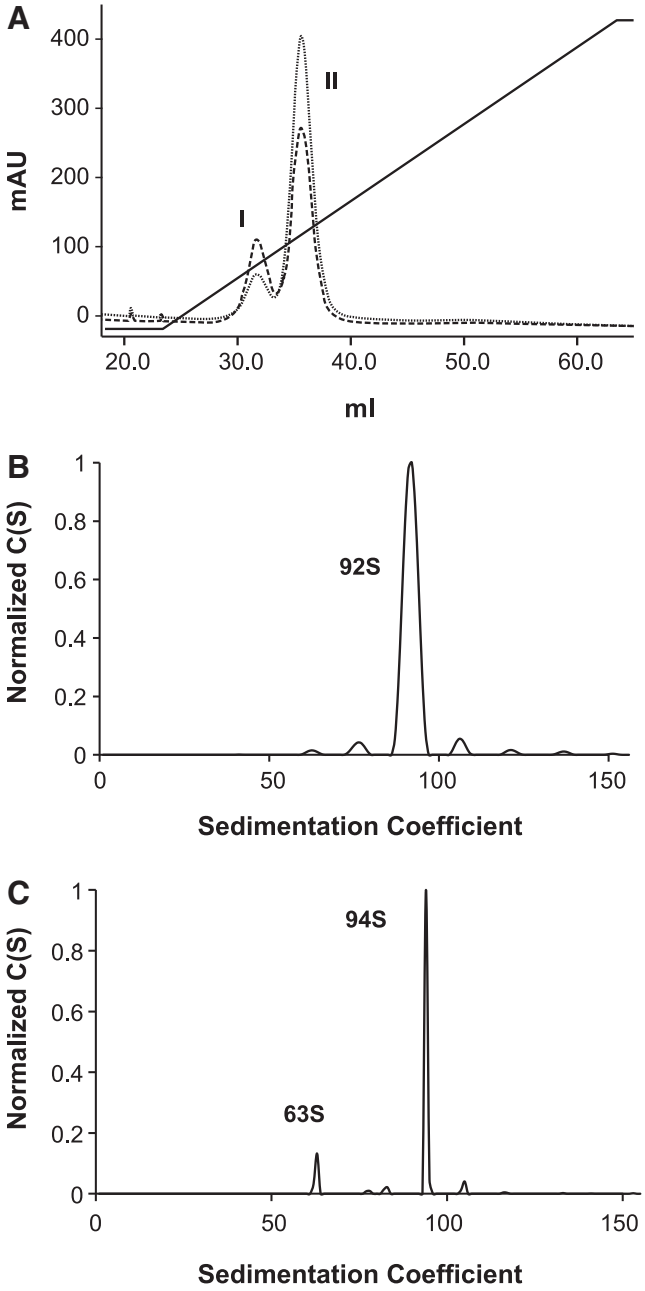


Figure 4. Separation of AAV2 particles by ion exchange chromatography. $AAV2_{3465}$ empty and genome-containing capsids were separated using ion exchange chromatography.[42] **(A)** Chromatogram following the elution of the column. Empty capsids (peak I) elute first, followed by genome-containing capsids (peak II). **(B)** Sedimentation distribution plots for peak II reveal that 94% of the capsids in this elution peak sediment with a sedimentation coefficient of 92S, representing genome-containing capsids. **(C)** Sedimentation distribution plots for the column load reveal two populations of capsids sedimenting at 63S and 94S, representing a mixture of empty and genome-containing capsids with a fractional content of 52% and 48%, respectively. A_{280} (- - - -) and A_{260} (……).

($R^2 = 0.9978$) between the sedimentation coefficient and genome size. Moreover, the relationship between sedimentation coefficient and genome size was shown to be independent of AAV capsid, supporting the use of the standard curve for AAV

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

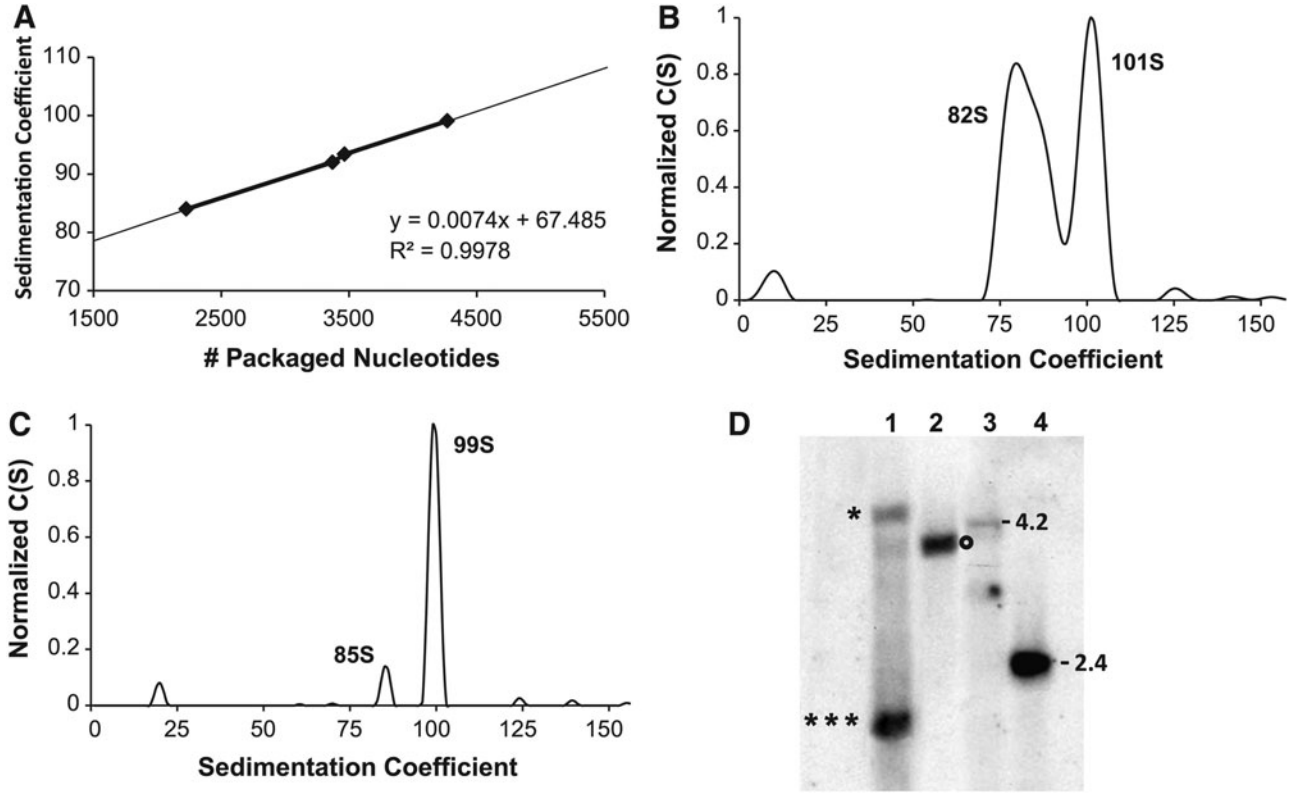


Figure 5. Correlation of vector genome size versus sedimentation coefficient. The sedimentation coefficient for a series of AAV vectors containing vector genomes of various sizes was determined. **(A)** These data were used to generate a standard curve revealing a high correlation between vector genome size and sedimentation coefficient. **(B)** Sedimentation distribution plots for a self-complementary AAV vector harboring a dimeric genome of approximately 4.3 kb and a monomeric genome of approximately 2.1 kb. The fractional content of capsids harboring the dimeric genome and sedimenting at 101S was 25%, whereas the fractional content of capsids harboring the monomeric genome and sedimenting at 82S was 75%. **(C)** Sedimentation distribution plots for a single-stranded AAV2 vector harboring a vector genome of approximately 4 kb; 84% of the capsid species is represented by a species sedimenting at 99S, and the remaining 16% is represented by a capsids species sedimenting at 85S. **(D)** Alkaline Southern blot analysis of the self-complementary AAV vector (lane 1) and the single-stranded AAV vector (lane 2). Lanes 3 and 4 represent the standards of 4.2 and 2.4, respectively. *, dimeric genome; ***, monomeric genome; 0, single-stranded vector genome.

vectors of any serotype. AUC analysis of different AAV vector serotypes, including AAV1, AAV2, AAV5, AAV6, AAV9, and AAVrh8R, harboring the identical *EGFP* vector genome, all generated sedimentation coefficient values of approximately 100S (Supplementary Fig. S1), suggesting that the encapsidated vector genome contributes more to the sedimentation coefficient (S value) than capsid proteins do. To demonstrate the utility of AUC to detect genome fragments, a self-complementary vector preparation of scAAV9*EGFP* was analyzed by AUC (Fig. 5B). The standard curve was then used to assign the genome size to each of the resolved genome-containing vectors, and approximately 25% of the vector preparation sedimented as a 101S species representing an encapsidated genome of approximately 4.3 kb (Fig. 5B). This 101S peak represents the double-stranded, dimeric vector genome that has a known size of approximately 4.3 kb. However, the majority of the vector preparation (75%) sedimented with an S value of 82S, which corresponds to a vector genome size of approximately 2.1 kb (Fig. 5B), consistent with the packaging of the single-stranded monomer.

The packaging of monomeric genomes, in the context of 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.[46] Figure 5D shows an alkaline Southern blot of the same scAAV9*EGFP* vector preparation, which revealed two vector population-containing genomes, approximately 4.3 and 2 kb in size, corroborating the AUC data in Fig. 5B. The Southern blot data also confirmed that the monomeric viral genome was preferentially packaged over the dimeric genome. Interestingly, AUC analysis of a single-stranded rAAV9*EGFP* vector revealed a single predominant peak (84%) with a measured S value of 99S, corresponding to

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

approximately 4200 nucleotides in the standard curve (Fig. 5C). Again, in agreement with the AUC method, Southern blot analysis of this vector preparation revealed homogeneous encapsidation of a viral genome approximately 4 kb in predicted size (lane 2, Fig. 5D). 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. These results also suggest that single-stranded rAAV vectors may be packaged in a more homogeneous manner than double-stranded self-complementary vectors.

Use of AUC to assess factors that influence packaging of vector genomes

The AUC method was next used to identify the factors that may influence the packaging of the

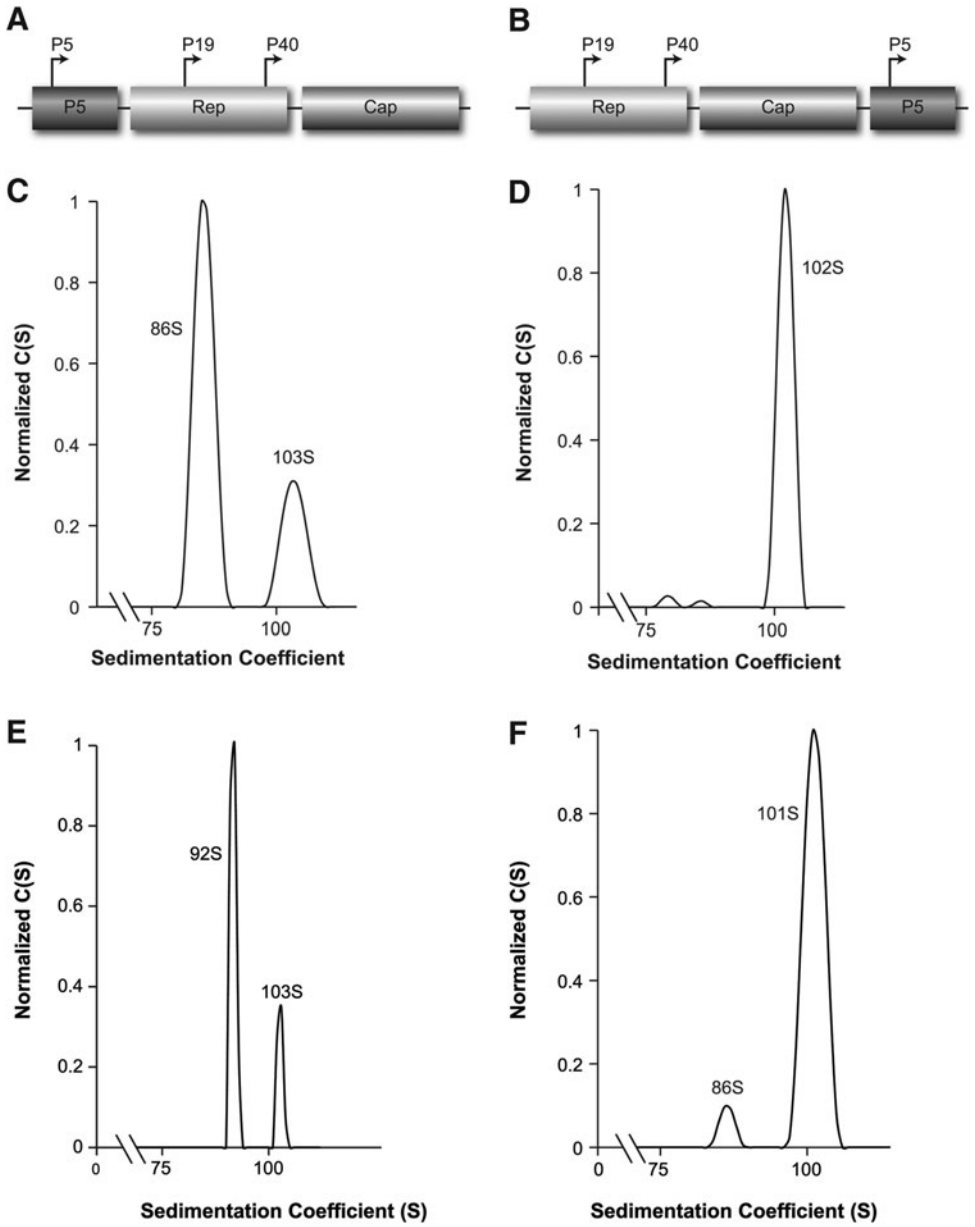


Figure 6. Schematic maps of the rep/cap helper plasmids used to generate the AAV vectors. **(A)** wt rep/cap helper with the p5, p19, and p40 endogenous promoters driving the expression of rep 68/78, rep 52/40, and cap, respectively. **(B)** pHLp rep/cap helper with the p5 promoter moved downstream of the cap ORF and a mutated TATA box. **(C, D)** Sedimentation distribution plots for an AAV5*FIX* vector harboring a vector genome of 4.4 kb produced using **(C)** wt rep/cap helper and **(D)** pHLp rep/cap helper. **(D)** In total, 94% of AAV5*FIX* vector capsids sediment at 102S. **(C)** Overall, 17% of the AAV5*FIX* vector capsids sediment at 103S and 83% sediment at 86S. **(E, F)** Sedimentation distribution plots for a repeat production of AAV5*FIX* produced using the wt rep/cap helper **(E)** or the pHLp rep/cap helper **(F)**. **(F)** In total, 90% of AAV5*FIX* vector capsids sediment at 101S and 10% sediment at 86S. **(E)** Overall, 17% of AAV5*FIX* vector capsids sediment at 103S and 83% at 92S.

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

intact AAV vector genomes. The production of rAAV vectors by transient transfection methods requires the use of three plasmids: an AAV rep/cap helper, an ITR transgene vector plasmid, and a pAd helper.[23] AUC was used to assess the effect of the rep/cap helper on vector genome packaging using one of two methods. In the first method (Fig. 6A), 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. 6B), the helper was modified such that rep 78/68 expression was reduced (data not shown), by moving the p5 promoter downstream of the cap sequence, and mutating the TATA box ("pHLp rep").[44] A single-stranded AAV5 vector encoding factor IX (AAV5*FIX*) and a single-stranded AAV5 vector encoding *SMN* (AAV5*SMN*) were generated using rep/cap helpers that differed in rep expression levels, as described.

Based on the size of the FIX expression cassette (4.4 kb), the predicted sedimentation coefficient for the AAV5*FIX* vector capsid was approximately 102S. AUC analysis of AAV5*FIX* made with pHLp rep revealed a homogenous profile with the majority of the vector (90%), sedimenting with an S value of the expected size, approximately 102S (Fig. 6D). By contrast, the AAV5*FIX* vector generated using the "wt rep," rep/cap helper, generated a strikingly different AUC profile (Fig. 6C). Instead of a predominant peak at 102S, the profile revealed more capsid heterogeneity, with the majority of the AAV5*FIX* (86%) sedimenting at a lower S value of 86S, likely representing packaging of a fragmented genome. Moreover, in this vector preparation, only 15% of the AAV5*FIX* vector sedimented at the correct S value of approximately 103S (Fig. 6C). A repeat production of the AAV5-*FIX* vector showed the same trend for improved vector genome packaging using pHLp rep (Fig. 6E and F). Similarly, AAV5*SMN* vectors, generated using these same rep/cap helpers, had different AUC profiles (Supplementary Fig. S2E and F). Similar to the AAV5*FIX* vectors, the AAV5*SMN* vectors generated with pHLp rep helper showed less heterogeneity by AUC analysis, with the majority of the vector sedimenting with an S value of 101S, consistent with packaging of a genome of the predicted size of 4.4 kb (Supplementary Fig. S2F). By contrast, the AUC profile for the same vector genome packaged using WT rep helper revealed three distinct AAV vector species, with sedimentation coefficients of 100S (predicted S value for the full vector genome of $\sim$4400 nt), 92S, and 80S (Supplementary Fig. S2E). Moreover, AAV5*EGFP* and $AAV5_{4600}$ generated using pHLP rep showed a similar trend for more homogenous packaging compared with the same vectors generated using WT rep (Supplementary Fig. S2A–D). Hence, for the vector sequences analyzed in this study, factor IX (*FIX*), survival motor neuron protein (*SMN*), and *EGFP*, using differing levels of rep 68/78 protein, can affect genome packaging efficiency.

Further analysis of the AAV5*SMN* and AAV5-*FIX* vector preparations was performed by Southern blotting. In agreement with the AUC method, Southern blot analysis of AAV5*SMN* generated with wild-type levels of rep 68/78 demonstrated packaging of full-length (4.4 kb) and fragmented (<4.4 kb) *SMN* genomes (Supplementary Fig. S3A, lane 1). By contrast, the AAV5*SMN* vector generated in the presence of pHLp rep predominantly contained vectors with a full-length *SMN* genome (Supplementary Fig. S3A, lane 2). Southern blot analysis of the AAV5*FIX* vectors generated under the two experimental conditions was also performed. The presence of a full-length *FIX* vector genome was confirmed for both vector preparations (Supplementary Fig. S3B, lanes 1 and 2); however, only the AAV5*FIX* vector generated in the presence of wild-type levels of rep 68/78 harbored a DNA fragment that was reactive with an *Amp*®-specific probe (Supplementary Fig. S3C, lane 2). This *Amp*-reactive 2.6 kb fragment is represented as an 86S species in the AUC profile, representing 80% of the total capsids (Fig. 6C). No *Amp*-containing fragment in the AAV5*FIX* vector preparations that were generated in the presence of pHLp rep was found (Supplementary Fig. S3C, lane 1). DNA impurities in the AAV5*FIX* preparations were also assessed by Q-PCR using primers and probes specific for *Amp*. The Q- PCR analysis of both AAV5*FIX* vector preparations corroborated the *Amp* Southern blot data, confirming that <1% *Amp* titer was detected in AAV5*FIX* vector preparations generated in the presence of pHLp rep. However, this finding is in contrast to an approximately 35% titer when the same vector plasmid was used to generate the AAV5*FIX* vector in the presence of wild-type rep 68/78. This result underscores the utility of AUC analysis in revealing the presence of packaged genomes that would be otherwise undetected by gene-specific Southern blot analysis.

Finally, a self-complementary AAV2*EGFP* vector was generated using rep-cap helpers that differed in the rep expression levels, as described above. In the presence of reduced rep 78/68 (pHLp

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

rep), more than half (56%) of the vector preparation contained dimeric genomes, as represented by the 101S species (Supplementary Fig. S4B). This result is the expected sedimentation coefficient for a capsid containing a dimeric genome of approximately 4300 nucleotides. By contrast, the scAAV2*EGFP* vector generated with wild-type levels of rep 78/68 had significantly fewer packaged dimeric genomes (26%), with the majority of the particles containing monomeric genomes and sedimenting at 82S (Supplementary Fig. S4A). Repeat production runs of the same vector comparing the two rep helpers yielded a similar result; scAAV2*EGFP* generated using pHLp rep contained over 50% dimeric genomes (Supplementary Fig. S4D) compared with approximately 30% with WT rep (Supplementary Fig. S4C). Similarly, production of a self-complementary $AAV9_{4500}$ vector, using either pHLp or WT rep, showed the same trend for improved packaging of the dimeric genome with pHLp rep (Supplementary Fig. S4E and S4F). The scAAV9 vector generated with WT rep contained 21% dimeric genomes (Supplementary Fig. S4E), in contrast to 56% when the same vector was generated with pHLp rep (Supplementary Fig. S4F).

DISCUSSION

Although rAAV vectors have emerged as an important class of novel biopharmaceutical drugs, robust methods to monitor the homogeneity, purity, and consistency of their manufacturing have not been developed. In an attempt to address this problem, we evaluated the potential of AUC as a technique to characterize the homogeneity of rAAV vectors. AUC analysis is highly versatile[12–18] and can be broadly applied to determine the biophysical properties of many types of particles, across a wide range of particle concentrations and sizes. We show that, by subjecting rAAV preparations to AUC under boundary sedimentation velocity conditions, the sedimentation of constituent rAAV capsids can be monitored at set time intervals. The differential sedimentation coefficient distribution value [C(S)] versus the sedimentation coefficient in Svedberg units (S) can be plotted, and the area under each peak in the C(S) distribution integrated to determine the relative concentration of each peak. Each peak represents a species of rAAV particle that is reflective of its molecular weight. The species that can be detected by this method include rAAV capsid particles comprising intact rAAV viral genomes (e.g., full virions), empty viral particles in which no rAAV viral genomes have been encapsidated, and rAAV viral particle variants in which variant rAAV viral genomes (e.g., truncated viral genomes) or nucleic acid contaminants are encapsidated. The genome size of a particular species of rAAV particles is calculated by comparing the S value of the species to a standard curve generated by S values of rAAV particles comprising encapsidated viral genomes of different known sizes. By resolving the genome-containing particles by size (e.g., dimeric and monomeric genomes or partial fragments), the AUC method represents a powerful tool to assay the quality of rAAV vector preparations produced under different conditions. Moreover, the results from distinct vector production systems demonstrated that the AUC method can be widely useful for quality control and the optimization of conditions to yield improved AAV vector preparations (Figs. 3, 4, and 6).

AUC analysis revealed that rAAV vectors produced using the standard triple transfection production protocol consist predominantly of empty particles. Moreover, we showed that the population of virions that contain DNA can be very heterogeneous. Furthermore, in addition to the full genome-containing vectors, a large proportion of the preparations contain fragmented genomes or non-genome-related DNA contaminants (Fig. 3). Improvements in vector quality are achieved when a producer cell line is used to generate rAAV vectors. With this platform, for certain vector genome sequences, the majority of the vectors produced contain full genome-containing particles (Fig. 3A and C), resembling the findings associated with wild-type AAV.[47] However, despite the preponderance of empty particles in transient transfection-produced rAAV vectors, chromatographic purification methods can be used to reduce their presence, and their removal can be monitored with AUC (Fig. 4).

Encapsidated plasmid DNA impurities have been shown to be derived from the backbone of the ITR-containing vector (cis) plasmid,[44] with the use of an oversized backbone in the vector production plasmid resulting in a significant reduction in plasmid-derived DNA impurities.[44] In this study, AUC showed that the inclusion of a large backbone alone may not be sufficient to minimize inadvertent backbone packaging and that rep protein (68/78) levels may also play a role (Fig. 6). *FIX* vector plasmid packaging, harboring a large backbone (~7 kb), in the presence of wild-type levels of rep 68/78 resulted in backbone packaging, as evidenced by AUC, Southern blot, and *AMP*-specific

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

TaqMan analysis, whereas backbone/*AMP* packaging was reduced in the presence of lower levels of rep proteins (Fig. 6 and Supplementary Fig. S3). Because vector plasmid replication is Rep68 mediated, a mechanism for encapsidation of plasmid backbone sequences has been proposed by Hauck et al.,[44] that is, a Rep68-mediated vector plasmid replication that inadvertently proceeds through the ITR junction and into the plasmid backbone.[44] Thus, the encapsidation of backbone/*Amp* sequences is likely reduced by using a helper expressing lower levels of the Rep68 protein. Similarly, when a series of vector plasmids with a smaller backbone ($\sim$2.5 kb) were packaged in the presence of reduced or wild-type levels of rep 68/78 proteins (Supplementary Fig. S2), remarkably different AUC profiles and Southern blots were revealed. In these examples, higher rep 68/78 protein levels resulted in increased packaging of fragmented vector genomes. Elevated rep 68/78 levels may contribute to the generation of fragmented genomes using a mechanism analogous to the mechanism reported for the rep 68 effects on self-complementary vector genomes, that is, spurious nicking at *trs*-like sites on the vector genome.[46] Imperfect fidelity in the AAV DNA-packaging mechanism[24] likely enables the inadvertent packaging of these fragmented genomes into preformed capsids. Additionally, using a single-molecule sequencing method, Kapranov et al.,[48] demonstrated that AAV packaging initiates from the 3′ end of the AAV ITR, whereas 5′ end mapping revealed premature termination as a mechanism that leads to incomplete vector genome packaging.

Self-complementary AAV vectors encoding a codon-optimized human FIX gene under the control of a liver-specific promoter have shown efficacy in preclinical models of hemophilia B and have also yielded exciting results in a phase 1 clinical trial, underscoring their utility in human gene therapy.[9] An optimal method to reliably quantitate the packaging of the dimeric self-complementary genome has not been described; therefore, we investigated the applicability of AUC in the characterization of self-complementary rAAV vectors. In agreement with Wu et al.,[46] we show that the packaging of dimeric self-complementary genomes (dsDNA) can be influenced by the level of rep proteins and that reducing the levels of rep68 shows a trend in an improvement in the dsDNA:ssDNA ratio (Supplementary Fig. S4). The applicability of the AUC method in the characterization of self-complementary vectors is significant. The many advantages that self-complementary vectors offer, including rapid onset of transgene expression[40] and increased biophysical stability,[49] may be offset by the challenge of reproducibly producing these vectors with the same degree of homogeneity, a requisite for all clinical vectors. Hence, the AUC method provides a quantitative measurement of the dsDNA:ssDNA ratio, providing a valuable tool for the quality assessment of clinical-grade self-complementary vectors.

In summary, we describe the development of a novel application of the AUC method to reveal the constituents of rAAV vector preparations, including empty vectors and vectors that contain complete or incomplete genomes. Typically, EM is used for the analysis of the ratios of empty AAV virions in viral vector preparations[49]; however, this method has limitations. Although EM technology is very useful and informative for qualitatively studying the structures of virions, this method is not ideal for the precise quantitative assessment of empty and full virions in a viral preparation. In addition, EM cannot distinguish full virions packaged with the full-length genome from virions with partial viral genomes or plasmid DNA impurities. Here, we show that AUC enables the precise and quantitative characterization of all constituent rAAV particles in real time. Moreover, this method can be used to assess factors that improve the quality of rAAV vector preparations. Vector genome integrity can be assessed by Southern blot analysis, and this method can reveal the range of encapsidated viral genomes; however, this technique cannot provide a quantitative assessment. Southern blotting relies on the manipulation of DNA before analysis and efficient reactivity with a probe of known sequence; thus, fragmented genomes and DNA impurities may go undetected because of the lack of complementarity with a vector sequence-specific probe. The ability to analyze all vector species is critical to assessing the homogeneity of clinical AAV vector preparations, which should predominantly consist of virions with a complete therapeutic genome. The presence of empty vectors and virions containing nongenomic nucleic acid contaminants is a concern because capsid proteins may trigger a T-cell-based immune response against the vector and the host cell transduced by the vector,[50] whereas encapsidated nongenomic nucleic acids can be transferred and persist *in vivo* after rAAV vector administration.[45] Additionally, vectors with fragmented genomes can behave as defective interfering particles and reduce the therapeutic potential of complete vec-

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

tors.[39] In conclusion, we have shown that AUC is a valuable method that should become a universal tool in the development of rAAV vectors to verify vector composition before clinical use. Importantly, AUC analysis of rAAV preparations enables the use of the same assay conditions to analyze different preparations of rAAV particles regardless of the rAAV genome nucleotide sequence and capsid serotype.

ACKNOWLEDGMENT

The authors are grateful to their colleague John Kingsbury of Genzyme, a Sanofi company, for technical advice.

AUTHOR DISCLOSURE

All authors are employees of Genzyme, a Sanofi company.

REFERENCES

1. Hauswirth WW, Alemean TS, Kaushal S, et al. Treatment of Leber congenital amaurosis due to a RPE65 mutation by ocular subretinal injection of adeno-associated virus gene vector short-term results of a phase I trial. Hum Gene Ther 2008; 19:979–990.
2. Bainbridge JW, Smith AJ, Barker SS, et al. Effect of gene therapy on visual frunction in Leber's congenital amaurosis. N Engl J Med 2008;358: 2231–2239.
3. Cideciyan AV, Aleman T, Boye SL, et al. Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow kinetics. Proc Natl Acad Sci USA 2008;105:15112–15117.
4. Cideciyan AV, Hauswirth WW, Aleman TS, et al. Human RPE65 gene therapy for Leber congenital amaurosis—persistence of early visual improvements and safety at 1 year. Hum Gen Ther 2009; 20:999–1004.
5. Cideciyan AV, Hauswirth W, Aleman TS, et al. Vision 1 year after gene therapy for Leber's congenital amaurois. N Engl J Med 2009;361:725–727.
6. Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med 2008;398:2240–2248.
7. Maguire AM, High KA, Aurricchio A, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: A phase I dose-escalation trial. Lancet 2009;374:1597–1605.
8. Simonelli F, Maguire AM, Testa F, et al. Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther 2010;18:643–650.
9. Nathwani AC, Tuddenham EG, Rangarajan S, et al. Adenovirus–associated virus vector mediated gene transfer in hemophilia B. N Engl J Med 2011;346: 2357–2365.
10. Yla-Herttuala S. Endgame: Glybera finally recommended for approval as the first gene therapy drug in the European Union. Mol Ther 2010; 20:1831–1832.
11. Ayuso E, Mingozzi F, Bosch F. Production, purification and characterization of adeno- associated vectors. Curr Gene Ther 2010;10:423–436.
12. Berkowitz SA, Philo J. Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation. Anal Biochem 2007;362:16–37.
13. Yang X, Agarwala S, Ravindran S, et al. Determination of particle heterogeneity and stability of recombinant adenovirus by analytical ultracentrifugation in CsCl gradients. J Pharm Sci 2006;97: 746–763.
14. Zhao H, Brautigam CA, Ghirlando R, Schuck P. Overview of current methods in sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation. Curr Protoc Protein Sci 2013; 20.12.1–20.12.49.
15. Schuck P. Size Distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys J 2000;78:1606–1619.
16. Philo JS. Analytical ultracentrifugation. In: Methods for Structural Analysis of Protein Pharmaceuticals. W Jiskoot, D Crommelin, eds. (AAPS, New York, NY). 2005; pp. 379–412.
17. Calabretta M, Jamison JA, Falkner JC, et al. Analytical ultracentrifugation for characterizing nanocrystals and their bioconjugates. Nano Lett 2005;5:963–967.
18. Zhou CM, Zhang GX, Ma XX. Characterization and evaluation of the immune responses elicited by a novel human papillomavirus (HPV) therapeutic vaccine: HPV16E7-HBcAg-Hsp65 fusion protein. J Virol Methods 2014;191–196.
19. Srivastava A, Lusby EW, Berns KI. Nucleotide seqeunce and organization of the adeno-assocaited virus 2 genome. J Virol 1983;45:555–564.
20. Sonntag F, Schmidt K, Kleinschmidt JA. A viral assembly factor promotes AAV2 capsids formation in the nucleolus. Proc Natl Acad Sci USA 2010; 107:10220–10225.
21. Grimm D, Kay MA, Kleinschmidt JA. Helper virus-free optically controllable and two plasmid–based production of adeno–associated virus vectors of serotypes 1 to 6. Mol Ther 2003;7: 839–850.
22. Matsushita T, Elliger S, Elliger C, et al. Adeno-associated virus vectors can be efficiently porduced without helper virus. Gene Ther 1998;5:938–945.
23. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol 1998;72: 2224–2232.
24. King JA, Dubielzig R, Grimm D, etal. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. EMBO J 2001;20:3282–3291.
25. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 2002;13:1935–1943.
26. Urabe M, Nakakura T, Xin K, et al. Scalable generation of high titer recombinant adeno-associated virus type 5 in insect cells. J Virol 2006;80:1874–1885.
27. Smith RH, Levvy J, Kotin RM. A simplified baculovirus AAV expression vector system coupled with one step affinity purification yields high-titer rAAV stocks from insect cells. Mol Ther 2009;17:1888–1896.
28. Chen H. Intron splicing mediated expression of AAV Rep and Cap genes and production of AAV vectors in insect cells. Mol Ther 2008;16:924–930.
29. Hwang KK, Mandell T, Kintner H, et al. High titer recombinant adeno-associated virus production using replication deficient herpes simplex virus type I. Mol Ther 2003;7:S14–S15.
30. Kang W, Wang L, Harrell H, et al. An efficient rHSV–based complementation system for the pro-

Downloaded by 163.116.146.117 from www.liebertpub.com at 07/21/23. For personal use only.

duction of multiple rAAV vector serotypes. Gene Ther 2009;16:229–239.

31. Clark KR, Voulgaropoulou F, Folet DM. et al. Cell lines for the production of recombinant adeno-associated virus. Hum Gene Ther 1995;6:1329–1341.

32. Gao GP, Qu G, Faust LZ, et al. High-titer adeno-associated viral vectors from a rep/cap cell line and hybrid shuttle virus. Hum Gene Ther 1998;9: 2352–2362.

33. Chadeuf G, Favre D, Tessier J, et al. Efficient recombinant adeno-associated virus production by a stable rep-cap HeLa cell line correlates with adenovirus induced amplification of the integrated rep-cap-genome. J Gene Med 2000;2:260–268.

34. Farson D, Harding TC, Tao L, et al. Development and characterization of a cell line for large scale, serum free production of recombinant adeno-associated viral vectors. J Gene Med 2004;6:1369–1381.

35. Thorne BA, Takeya RK, Peluso RW. Manufacturing recombinant adeno associated viral vectors from producer cell lines. Hum Gene Ther 2009;20:707–714.

36. Yuan Z, Qiao C, Hu P, et al. A versatile adeno-associated virus vector producer cell line method for scaleable vector production of different serotypes. Hum Gene Ther 2011;22:613–624.

37. Martin J, Frederick A, Luo Y, et al. Generation and characterization of adeno-associated virus producer cell lines for research and preclinical vector production. Hum Gene Ther Meth 2013;24:253–269.

38. Satkunanathan S, Wheeler J, Thorpe R, et al. Establishment of a novel cell line for the enhanced production of recombinant adeno-associated virus vectors for gene therapy. Hum Gene Ther 2014;25: 1–13.

39. Gao K, Li M, Zhong L, et al. Empty virions in AAV8 vector preparations reduce transduction efficiency and may cause total viral particle dose-limiting side effects. Mol Ther Meth Clin Dev 2014;1: 9–13.

40. McCarty DM, Monahan PE, Samulski RJ. Self complementary recombinant adeno-associated virus (scAAV) vectors promote efficienct transduction independently of DNA synthesis. Gene Ther 2001;16:1248–1254.

41. De La Maza LM, Carter BJ. Heavy and light particles of adeno associated virus. J Virol 1980;33: 1129–1137.

42. Qu G, Bahr-Davidson J, Prado J, et al. Separation of adeno-assocaited virus type 2 empty particles from genome containing vector by anion–exchange chromatography. J Virol Methods 2007;140:183–192.

43. Sommer JM, Smith PH, Parthasarathy S, et al. Quantification of adeno-assocaited virus particles and empty capsids by optical density measurement. Mol Ther 2003;7:122–128.

44. Hauck B, Murphy SL, Smith PH, et al. Undetectable transcription of *cap* in a clinical AAV vector: Implications for preformed capsid in immune responses. Mol Ther 2009;17:144–152.

45. Chadeuf G, Ciron C, Moullier P, et al. Evidence for encapsidation of prokaryotic sequences during recombinant adeno-assocaited virus production and their *in vivo* persisitence after vector delivery. Mol Ther 2005;12:744–753.

46. Wu JW, Zhao L, Zhang Z, et al. Self-complementary recombinant adeno-assocaited viral vectors: packaging capacity and the role of the rep proteins in vector purity. Hum Gene Ther 2007;18:171–182.

47. Zeltner N, Kohlbrenner E, Clement N, et al. Near-perfect infectivity of wild type AAV as benchmark for infectivity of recombinant AAV vectors. Gene Ther 2010;17:672–679.

48. Kapranov P, Chen L, Dederich D, et al. Native molecular state of adeno-associated viral vectors revealed by single-molecule sequencing. Hum Gene Ther 2012;23:46–55.

49. Horowitz ED, Rahman KS, Bower BD, et al. Biophysical and ultrastructural characterization of adeno-associated virus capsid uncoating and genome release. J Virol 2013;87:2994–3002.

50. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. Nat Med 2006;12:342–347.

Received for publication April 27, 2015;
accepted after revision September 14, 2015.

Published online: September 17, 2015.