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Quantification of Adeno-Associated Virus Particles and Empty Capsids by Optical Density Measurement

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We show here that UV absorbance of denatured adeno-associated virus (AAV) vector provides a simple, rapid, and direct method for quantifying vector genomes and capsid proteins in solution. We determined the molar extinction coefficients of capsid protein to be 3.72×10^6 M^{-1} cm^{-1} at 260 nm and 6.61×10^6 M^{-1} cm^{-1} at 280 nm. For recombinant AAV vectors, extinction coefficients can be calculated by including the predicted absorbance of the vector DNA. Since the amount of empty capsids in purified vector preparations lowers the A_{260}/A_{280} ratio in a predictable manner, the vector genome (vg) and capsid particle (cp) titers in purified AAV vector preparations can be calculated from the absorbance at 260 nm and the A_{260}/A_{280} ratio. To validate this method, the vg and cp titers calculated by UV absorbance were compared with titers determined by quantitative (Q)-PCR and capsid ELISA. The vg titers determined by absorbance agreed well with titers determined by Q-PCR. The cp/vg ratio determined by the A_{260}/A_{280} method also correlated well with those determined by AAV capsid ELISA in conjunction with Q-PCR. This new method provides a simple and rapid means to determine AAV vg titers and the ratio of empty to full particles in purified virus preparations.

Key Words: adeno-associated virus, spectrophotometry, extinction coefficient, real-time quantitative PCR, capsid ELISA

INTRODUCTION

Adeno-associated virus (AAV)-based vectors show increasing promise in gene therapy due to their relative safety and long-term gene expression [1,2]. Methods for large-scale production of highly purified AAV for clinical use have improved considerably in recent years [3–6]. Quantification of AAV vector products depends on accurate measurements of packaged vector genomes by quantitative (Q)-PCR or dot-blot hybridization, which frequently are the dose-defining assays for clinical use. Optical density has long been used for quantification of adenovirus. The extinction coefficient of adenovirus type 2 was determined empirically by Maizel *et al.* [7]. The conditions for vector denaturation and spectroscopy were further optimized by Mittereder *et al.* [8] and Sweeney and Hennessey [9]. Adenovirus contains 12–14 different proteins [10], and its extinction coefficient may be difficult to predict from first-order principles. The relatively simple structure of AAV, on the other hand, allowed us to predict the absorbance based on the number of aromatic amino acids contained in the three capsid proteins, and the results confirmed our experimental determination of the extinction coefficient by amino acid analysis.

AAV vectors are composed of a single-stranded DNA genome and three well-characterized proteins. These viral proteins, VP1, VP2, and VP3, are derived from a single open reading frame by alternate splicing [11]. The high-resolution structure of AAV type 2 has recently been determined by X-ray crystallography [12]. Based on this and other studies, each viral particle appears to be composed of 5 VP1, 5 VP2, and 50 VP3 molecules, which allowed us to accurately estimate the molecular weight of the capsid structure based on the known sequences of VP1, VP2, and VP3. Empty capsids that are typically formed during AAV vector production copurify with genome-containing vector particles during chromatographic purification, and the excess of empty capsids confounds simple determinations of vector genome concentration by absorbance. The method described here takes into account the capsid ratio, deduced from the A_{260}/A_{280} ratio, to determine vector genome (vg) concentrations. This approach resulted in a simple, rapid, and accurate quantification method for vector genomes at concentrations above 5×10^{11} vg/ml

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in the presence of up to 40-fold excess empty capsids. We show here that the results from the absorbance assay correlate well with the vector genome and capsid values determined by Q-PCR, dot blot, ELISA, and electron microscopy in CsCl density gradient and column-purified vector preparations.

RESULTS

Extinction Coefficient of AAV2 Capsid Protein

Quantitative amino acid analysis is an accurate method for determining protein concentration in solution and has been used to determine the extinction coefficients of proteins [13]. We used a preparation of highly purified empty AAV2 capsids, designated LN 431.92, to determine the molar extinction coefficient of capsids. This lot of AAV particles was produced by transfection of 293 cells with plasmids encoding the AAV *rep* and *cap* genes and adenovirus helper functions but without a template for the vector genome. Under these conditions, empty particles are assembled and can be purified to homogeneity by column chromatography. Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–PAGE) confirmed that this preparation had no detectable protein impurities and 99.6% of the particles appeared empty by electron microscopy (data not shown). Amino acid analysis indicated an amino acid composition that accurately reflected the predicted mixture of VP1, VP2, and VP3 proteins to within 10% of the expected relative amounts for every amino acid. Based on the quantification of each amino acid, the sample contained 310 μg/ml protein. This sample had an average absorbance of 0.523 at 280 nm, with an A_{260}/A_{280} of 0.589. From these results, we calculated the extinction coefficients (ϵ) for AAV2 capsid of $\epsilon_{260(capsid)} = 3.72 \times 10^6\ M^{-1}\ cm^{-1}$ and $\epsilon_{280(capsid)} = 6.61 \times 10^6\ M^{-1}\ cm^{-1}$. The known structure of AAV allowed us to predict the absorbance based on the number of chromophores in the amino acid sequence of the three capsid proteins, using the extinction coefficients for denatured VP1, VP2, and VP3 as predicted by ProtPharm software (Expert Protein Analysis System, Proteomics server of the Swiss Institute of Bioinformatics), which is based on the equations developed by Gill and von Hippel [14]. We neglected minor contributions to the UV absorbance from potential cystine residues and assumed that there are no prosthetic groups or posttranslational protein modifications that affect the absorbance at 260 or 280 nm. This theoretical approach gave a value of $\epsilon_{280(capsid)} = 6.23 \times 10^6\ M^{-1}\ cm^{-1}$ for AAV2 capsids, which differs by <6% from the value determined experimentally.

AAV Particle Denaturation

Our method for measuring the UV absorption of AAV capsid protein assumes that the chromophores of all aromatic amino acids in the peptide sequence contribute to the overall absorption. This requires that the chromophores be exposed to an aqueous, neutral environment and not perturbed by the general local environment or electrostatic interactions with nearby chromophores. Complete denaturation of vector protein prior to the UV measurement ensures optimal absorbance properties for every chromophore and was necessary to prevent the Rayleigh light scattering that is observed for intact AAV particles in the UV range. We used dynamic light scattering to assess disruption of AAV particles. In preliminary experiments it was determined that even relatively high concentrations of SDS (2%) are not sufficient to disrupt AAV at room temperature or 37°C; however, heating to 75°C in 0.05% SDS for 5 min was determined to result in complete disruption of AAV particles at concentrations up to 10^{14} capsid particles (cp)/ml. Incubation times up to 2 h or higher SDS concentration and higher temperatures did not further alter the absorbance, suggesting that the proteins are completely denatured (data not shown). We chose conditions of 0.1% SDS and 10 min at 75°C for our routine sample treatment prior to absorbance measurement.

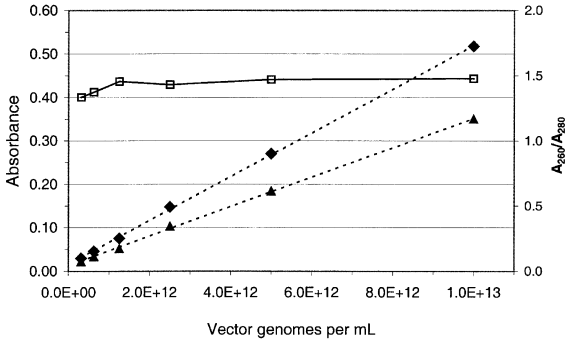

FIG. 1. UV absorbance of denatured AAV vector. An aliquot of AAV-hFIX16 (RVC 257, CsCl density gradient purified) at a starting concentration of 10^{13} vg/ml was diluted in PBS–5% sorbitol as a twofold dilution series and denatured in 0.1% SDS at 75°C for 10 min. The absorbance was measured in a spectrophotometer at 260 nm (◆) and 280 nm (▲). The expected absorbance ratio for highly purified AAV-hFIX16 without any empty particles is 1.44. The observed absorbance ratio (□) remained relatively constant at 1.42 ± 0.04 standard error over the quantification range from 2.5×10^{11} to 10^{13} vg/ml.

Specificity, Linearity, and Range

Accurate measurement of vector absorbance depends on an appropriate blank that reflects the vector formulation. When formulated in PBS or Tris-buffered saline, concentrations as low as 5×10^{11} vg/ml resulted in reproducible OD readings. We observed a linear absorbance range to at least 10^{13} vg/ml (Fig. 1). Residual protein impurities, as well as excipients that absorb in the UV range such as Tween 80 or bovine serum albumin (BSA), may interfere in this assay. However, these additives are often used to prevent loss of material by adsorption to storage containers. As an alternative surfactant to Tween 80, we used

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Pluronic F-68, which does not show measurable absorbance at 260 and 280 nm, but prevents loss of AAV due to nonspecific adsorption during storage and vector manipulations at concentrations as low as 0.001%.

Vector Genome Concentration

The absorbance (A_{260}) of a highly purified AAV preparation depends on the MW of the vector DNA and the amount of capsid protein. All experiments described in this report were performed with AAV-hFIX16, which contains a "minigene" derived from the human clotting factor IX (FIX) controlled by liver-specific enhancer and promoter elements. The calculated molecular weight of the vector genome (MW_{DNA}) is 1.33×10^6 g/mol. Both the positive and the negative strand of AAV vector DNA are packaged at similar frequencies [15]. We reasoned that the vector genomes anneal to form double-stranded DNA (dsDNA) following denaturation of AAV. To test this assumption, we quantified DNA by the dsDNA-specific dye PicoGreen after heat/SDS treatment and a 20-min incubation at room temperature. This method indicated an amount of dsDNA that was 136 ± 28% of the expected amount of vector DNA, suggesting that the majority of vector DNA had indeed annealed. Without heat treatment, or when assaying an equivalent amount of single-stranded control DNA (M13), PicoGreen detected only 10 to 30% of the DNA present. We therefore used the extinction coefficient of dsDNA for calculation of vector genomes in this assay. For samples containing only full vector, the capsid protein contributes 12% to the absorbance at 260 nm, and the vector quantification by OD is thus not very sensitive to minor amounts of empty capsids. At a 10-fold excess of empty capsids, the protein contributes 60% of the absorbance at 260 nm. Equation (6) presented under Materials and Methods was used to calculate vg/ml from the A_{260} and A_{280} in eight AAV vector lots of hFIX16, with results ranging from 5.17×10^{11} to 1.06×10^{13} vg/ml. These results compared well with the vg titers determined by Q-PCR, with a correlation coefficient of 0.985 and a slope of 1.10 (Fig. 2). On average, the vector genome results obtained by absorbance differed by 8.2% from the Q-PCR results (Table 1). Four of these lots were also characterized by dot-blot hybridization, with similar agreement between the vg results from all three methods (Table 1).

Estimation of Empty Capsid Content in Vector Preparations

Based on Eq. (4) given under Materials and Methods using $MW_{DNA} = 1.33 \times 10^6$ g/mol, the A_{260}/A_{280} for a preparation of 100% full AAV-hFIX16 vector particles (cp/vg = 1) is 1.438 and that of 100% empty capsids is 0.590. For vector preparations that contain a mixture of empty and full particles, such as those purified by column chromatography, the cp/vg ratio can be deduced from the observed absorbance ratio. Figure 3 shows the predicted relationship between cp/vg and A_{260}/A_{280}. The shape of the curve indicates that the absorbance ratio can be used to estimate the capsid ratio for values from cp/vg = 1 (no empty capsids) to approximately 20. Beyond that, the absorbance ratio approaches the asymptotic value for pure capsid protein (0.590) and the determination of the capsid ratio is less precise.

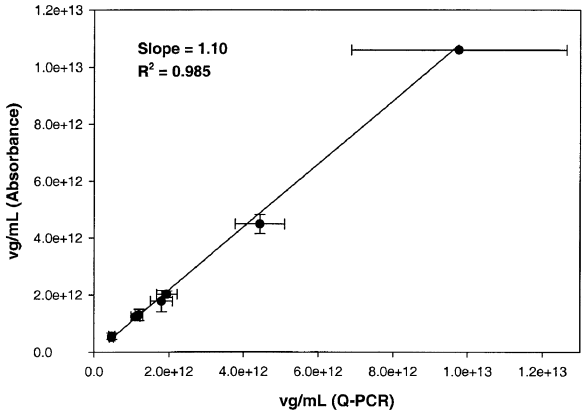

FIG. 2. Correlation of vector genome concentration determined by absorbance vs quantitative PCR. Eight lots of AAV-hFIX16 with capsid to vector genome ratios ranging from 1 to 40 and titers ranging from 4.6×10^{11} to 1×10^{13} vg/ml were analyzed by spectrophotometry. The calculated vg/ml based on the absorbance is plotted against the vg/ml obtained by Q-PCR. Error bars indicate the standard deviations between replicate assays.

We determined cp/vg ratio based on A_{260}/A_{280} for eight lots of AAV-hFIX16 vector and two lots of empty particles (Table 1). The vector samples included one lot prepared by CsCl gradient density centrifugation (RVC 257) and seven lots of column-purified vector. The cp/vg ratios were also determined by ELISA and Q-PCR, and comparison of the results is shown in Fig. 4. The absorbance method correctly predicted a cp/vg ratio of 1.0 for the CsCl-gradient-purified vector (RVC 257), which had less than 1% empty particles by electron microscopic analysis. The capsid ratio determined by ELISA/Q-PCR was 0.90 for this lot (Table 1). For the seven column-purified vector samples, the cp/vg ratio as determined by absorbance ranged from 10 to 34 and agreed well with the values determined by ELISA/Q-PCR, as indicated by a correlation coefficient of 0.995 and a slope of 1.02 over this range (Fig. 3). Five AAV-hFIX16 lots were also characterized by electron microscopy (EM), by which empty and full particles can be counted. The absorbance results correlated well with the EM, with two notable exceptions, DCL 1 and DCL 3 (Table 1), which had significantly lower ratios by EM analysis than by OD or capsid ELISA. Overall, the correlation (R^2) of capsid ratios determined by EM vs OD in vector samples was 0.96, including DCL 1 and DCL 3.

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TABLE 1: Comparison of capsid ratios and vector genome titers determined by absorbance, electron microscopy, Q-PCR, ELISA, and dot blot

Sample	A_{260}	A_{260}/A_{280}	cp/vg (absorbance)	cp/vg (EM)	cp/vg (Q-PCR & ELISA)	vg/ml (absorbance)	vg/ml (Q-PCR)	vg/ml (dot blot)
RCV 257 (CsCl)	0.5330	1.4450	0.97	1.0	0.90	1.06×10^{13}	$9.76 \times 10^{12} \pm 2.88 \times 10^{12}$	nd
DCL 1 (column)	0.2110 ± 0.0098	0.7200	19.4 ± 3.2	10.3	20.6 ± 3.4	$1.30 \times 10^{12} \pm 1.97 \times 10^{11}$	$1.20 \times 10^{12} \pm 1.03 \times 10^{11}$	$1.14 \times 10^{12} \pm 2.4 \times 10^{11}$
DCL 2 (column)	0.1976 ± 0.0211	0.8033	11.0 ± 1.7	9.5	9.7 ± 0.4	$1.78 \times 10^{12} \pm 3.72 \times 10^{11}$	$1.80 \times 10^{12} \pm 2.95 \times 10^{11}$	$1.65 \times 10^{12} \pm 2.1 \times 10^{11}$
DCL 3 (column)	0.1760 ± 0.0014	0.7450	15.8 ± 0.1	11.2	16.6 ± 3.9	$1.24 \times 10^{12} \pm 1.41 \times 10^{10}$	$1.11 \times 10^{12} \pm 1.22 \times 10^{11}$	1.20×10^{12}
DCL 4 (column)	0.2115 ± 0.0091	0.8220	9.8 ± 0.2	8.3	9.7 ± 0.5	$2.02 \times 10^{12} \pm 1.13 \times 10^{11}$	$1.94 \times 10^{12} \pm 2.75 \times 10^{11}$	$1.99 \times 10^{12} \pm 9.8 \times 10^{10}$
Q 1 (column)	0.5255 ± 0.0290	0.7890	11.8 ± 0.4	nd	11.0 ± 1.7	$4.49 \times 10^{12} \pm 3.32 \times 10^{11}$	$4.44 \times 10^{12} \pm 6.62 \times 10^{11}$	nd
Q 2 (column)	0.1320	0.6670	33.8	nd	30.6 ± 6.3	5.17×10^{11}	$4.64 \times 10^{11} \pm 5.10 \times 10^{10}$	nd
Q 3 (column)	0.1325 ± 0.0106	0.6735	31.3 ± 4.5	nd	31.1 ± 3.9	$5.60 \times 10^{11} \pm 1.12 \times 10^{11}$	$4.75 \times 10^{11} \pm 8.22 \times 10^{10}$	nd
RVC 189c (empty)	0.7660	0.6080	143	267	nd	na	na	na
RVC 257-E (empty)	0.1672	0.6278	69.2	143	nd	na	na	na

nd, not determined; na, not applicable.

DISCUSSION

The assay presented in this report is a simple, rapid, and accurate method to measure capsid particle and vector genome concentrations in solutions of purified AAV virus or vectors. The assay does not require a standard curve and uses equipment that is readily available in most laboratories. Moreover, the assay provides both the total capsid concentration and the specific vector yield, which are important parameters during AAV manufacture. Previously, two assays were needed to determine these parameters independently, both of which are relatively laborious and time consuming.

Biosynthesis and assembly of AAV results in the formation of empty capsids that lack a viral genome. The pro-

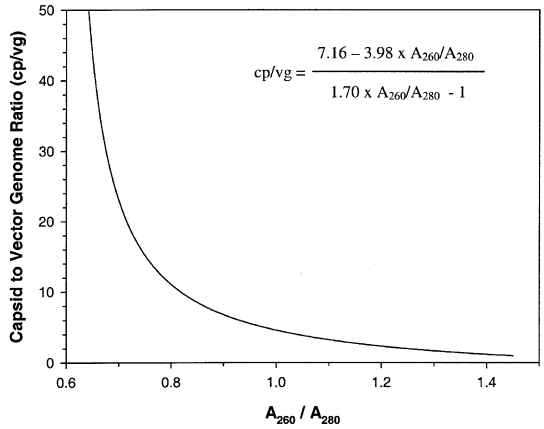

FIG. 3. Predicted relationship of capsid to vector genome ratio (cp/vg) and absorbance ratio (A_{260}/A_{280}) for AAV-hFIX16. The theoretical A_{260}/A_{280} ratio was plotted for the recombinant AAV-hFIX16 vector with a cp/vg ratio ranging from 1 (no empty capsids) to 50 (98% empty capsids).

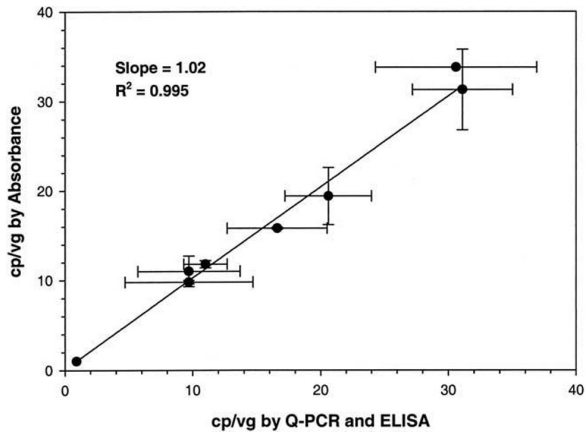

FIG. 4. Comparison of absorbance measurement and Q-PCR/ELISA for determining capsid to vector genome ratio. The cp/vg ratios derived from absorbance measurements and ELISA are shown for eight samples of AAV-hFIX16. Error bars indicate the standard deviations between replicate assays.

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portion of empty to full particles depends primarily on the size and sequence of the vector genome to be packaged, but also on the cell culture system and mode of AAV production and purification (unpublished). We have observed a range from less than 50% empty capsids in cell harvests for AAV2 wild type to over 98% for certain recombinant AAV vectors that package poorly. While traditional methods of AAV purification based on density gradient centrifugation separate empty AAV capsids from genome-containing vector particles, most scalable chromatography-based methods for vector purification performed do not achieve this separation. The absorbance method to determine AAV capsid particle and vector concentrations reported herein is applicable to density gradient-purified and column-purified vectors. Knowing the total AAV capsid protein content of a vector preparation becomes important as production yields and concentration requirements increase and concerns about possible vector aggregation arise [16]. Overall, our results suggest that the absorbance assay can predict capsid ratios about as well as the combined use of capsid ELISA and Q-PCR, in which the cumulative variability of the two assays has to be taken into consideration when determining this ratio.

One limitation of this method is the need for relatively high concentrations of purified AAV ($>5 \times 10^{11}$ vg/ml). Another limitation is that significant protein or nucleic acid impurities, as well as buffers or excipients that absorb in the UV range, can affect the accuracy of the results. An appropriately matched formulation buffer, used as an assay blank, may overcome some of the effects of excipients, but vector purity needs to be verified independently. To test the effects of protein impurities in this assay, BSA was spiked into a sample of column-purified vector (cp/vg = 15) at one-half of the mass of capsid protein to mimic a protein impurity of approximately 30%. This resulted in a 14.5% increase in the apparent cp/vg ratio and had a negligible effect on the vg concentration. The specific absorption of BSA at 280 nm is approximately 2.5-fold lower than that of AAV capsid protein (by mass), and the observed effect of adding BSA was consistent with an expected 20% increase in apparent capsid concentration.

The studies reported here have demonstrated the usefulness of spectrophotometry in quantifying vector particles and empty capsids for a specific AAV2-derived vector. The equations provided under Materials and Methods can be used to calculate the expected absorbance of AAV2-based vectors with different genome molecular weights. Related AAV serotypes differ slightly in their capsid composition, which may result in different extinction coefficients. Our calculations based on the capsid sequences deposited at GenBank suggest that the predicted extinction coefficients at 280 nm would be 1 to 2% lower for AAV3 (AAB95452) and AAV6 (AAB95450) and 4.5% lower for AAV5 (AAD13756) than for the AAV2 serotype presented in this study. We therefore anticipate that the absorbance assay will require only minor modification when applied to other AAV serotypes.

MATERIALS AND METHODS

AAV purification. AAV vectors were generated by triple transfection of HEK-293 cells [17] and purified by column chromatography as described previously [18]. Briefly, cell harvests containing AAV were microfluidized and filtered through 0.2-μm filters. The AAV was purified from the clarified cell lysates by sequential cation- and anion-exchange chromatography, concentration by ultrafiltration, and diafiltration into phosphate-buffered saline containing 5% sorbitol, pH 7.4 (PBS–sorbitol). Where indicated, empty capsids were removed by CsCl density gradient centrifugation and fractions of a density from 1.38 to 1.42 were collected. The pooled fractions were dialyzed against PBS–sorbitol. In some cases, 0.01% Pluronic F-68 (BASF Corp., Mount Olive, NJ), which has no impact on the UV absorbance, was added to prevent vector loss. Purity was assessed by SDS–PAGE. Purified AAV vector used in this study essentially showed only VP1, VP2, and VP3 by silver staining of SDS–PAGE gels. Residual protein impurities were also measured by ELISAs specific for HEK-293 host cell protein (HCP) or BSA in several vector lots (DCL 1, 2, 3, and 4). Both HCP and BSA amounted to less than 0.1% of total protein. Residual host cell (mammalian) DNA and plasmid DNA, as determined by specific Q-PCR analysis, were less than 1% of the viral DNA (data not shown).

RVC 257 was purified by column chromatography followed by two consecutive CsCl density gradients to ensure complete removal of empty capsids. RVC-257-E was prepared by collecting the empty capsids band (density 1.31) from the first CsCl gradient during the production of RVC 257. RVC 189 lacked vector template during manufacture and therefore assembled only empty capsids. The empty capsids were purified by column chromatography, followed by an additional purification and concentration on a second cation-exchange column. RVC 189c was the most concentrated fraction collected during the additional purification and before pooling several fractions to produce lot 431.92.

Real-time quantitative PCR. The vectors used in this study are designated AAV-hFIX16 and contain the human factor IX "minigene" [19] fused to liver-specific promoter and enhancer elements. The Q-PCR primers and probe anneal to exons 5 and 6 of the FIX gene, thus spanning an intron not present in the vector sequence and thereby minimizing amplification of genomic DNA. Real-time Q-PCR [20] was standardized with plasmid DNA containing this vector insert (pAAV-hFIX16). The plasmid was linearized with a restriction enzyme, purified, quantified by UV absorbance, and diluted in Q-PCR dilution buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10 μg/ml yeast tRNA, and 0.1% Tween 80) to result in eight standards ranging from 61 to 10^6 copies per reaction, assuming a MW of 6.97×10^6 Da for pAAV-hFIX16. Each standard was run in four replicate 50-μl reactions in alternate rows of a 96-well optical plate. For each Q-PCR sample, three dilutions (100-, 300-, and 1000-fold diluted in Q-PCR dilution buffer) were added to DNase buffer (10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, and 10 units DNase I) as a 5-fold dilution and incubated at 37°C for 30 min. DNase was inactivated and the vector DNA was solubilized by addition of an equal volume of 200 mM EDTA, pH 8.0, and an incubation at 95°C for 20 min. After a further 100-fold dilution in Q-PCR dilution buffer, 10 μl of sample was added to four replicate Q-PCR wells containing 40 μl reaction mix in alternate rows. The PCR was carried out on an Applied Biosystems 7700 Sequence Detection System. Vector genome titers were calculated by multiplying the copies per well with the above dilution factors and assuming that one copy of double-stranded plasmid DNA is equivalent to two single-stranded vector genomes.

Dot blot. Where indicated, the vector genome concentration was determined by quantitative dot-blot hybridization. DNA was extracted from vector samples, denatured, and bound to nitrocellulose. A portion of the factor IX minigene was gel purified and radiolabeled with ^{32}P by random priming as a probe. The hybridization signal from bound vector DNA was compared to that of plasmid DNA standards (pAAV-hFIX16) bound to the same membrane by quantitative phosphorimaging.

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PicoGreen assay. Vector DNA or M13 single-stranded control DNA was quantified using the PicoGreen dsDNA Quantification Kit (Molecular Probes, Inc., Eugene, OR). An aliquot of AAV-hFIX16 (LN DCL3) was diluted 2-fold and treated with 0.1% SDS at 75°C for 10 min. The sample was cooled to room temperature for 20 min and then diluted 50-fold with TE. Equal volumes of diluted denatured vector were mixed with PicoGreen reagent and the fluorescence was measured in a multititer plate reader. The assay was standardized with dsDNA included in the assay kit.

Capsid ELISA. The capsid concentration was determined using a commercially available ELISA kit (American Research Products, AAV Titration ELISA, Cat. No. PRATV). Multititer strips were coated with anti-AAV2 monoclonal antibody specific for assembled capsids. Captured AAV particles were detected with biotinylated anti-AAV2 antibody and HRP-conjugated streptavidin [21]. The assay was standardized with AAV-hFIX16 (RVC 257) that had been purified by column chromatography followed by two sequential CsCl density gradient centrifugation steps to obtain essentially 100% full (vector-genome-containing) vectors, as confirmed by electron microscopy. The titer of the diluted standard (LN 431.96), and thus the capsid concentration, was determined as the average of eight independent Q-PCR assays.

Electron microscopy. Samples of purified vector were applied to carbon-coated electron microscopy grids, negatively stained with 2% uranyl acetate, and examined by transmission electron microscopy at 60,000-fold enlargement. Empty capsids can be readily distinguished from full capsids as doughnut-shaped particles, resulting from the uptake of uranyl acetate in the core of the capsid or from collapse and differential staining of the particle. A brief drying step and rinse with distilled water before the staining enhanced the distinct appearance of empty particles. For determining the empty and full capsid ratios, a minimum of 300 viral particles were counted, distributed over several representative electron micrographs.

Quantitative amino acid analysis. Amino acid analysis was carried out on a preparation of highly purified empty capsids (LN 431.92). The empty capsid preparation was made by producing AAV in the absence of vector genomes, allowing assembly of only genome-less particles. Assembled particles were purified by chromatography as for AAV vector, followed by a further purification and concentration step on an additional cation-exchange column. The purity of empty capsids and absence of DNA-containing particles in this preparation were confirmed by electron microscopy, which showed 99.6% of the particles with a doughnut-shaped appearance characteristic of empty particles. AppTec Laboratory Services (Camden, NJ) performed the amino acid analysis on a hydrolyzed sample of LN 421.92 by fluorescence derivatization and quantitative detection of all amino acids (except tryptophan) by reverse-phase HPLC.

Absorbance measurement. UV spectrophotometry of intact viral particles results in significant light scatter due to the Rayleigh effect [22]. The virus particles therefore need to be dissociated into DNA and protein subunits. Vector samples were denatured before the absorbance measurement by adding 0.5 μl of a 20% stock solution of SDS to 100-μl sample or blank (formulation buffer) in polypropylene Eppendorf tubes, followed by heat treatment at 75°C for 10 min. Both blank and samples were processed in parallel to compensate for any potential leaching of UV-absorbing material from the tubes. All samples and blanks were assayed in the same quartz microcuvette on an Agilent Model 8453 diode array spectrophotometer, with readouts at 260 and 280 nm. Absorbance values above 0.025 AU were considered sufficiently reliable to be included in this assay.

Calculation of capsid ratio and vector genome concentration. The theoretical molecular weight of an AAV serotype 2 capsid is based on the published sequences of the three viral proteins VP1, VP2, and VP3 (GenBank Accession Nos. AAC03780, AAC03778, and AAC03779) and the three-dimensional structure obtained by X-ray crystallography [12]. Accordingly, a vector particle, which is composed of 5 molecules of VP1, 5 VP2, and 50 VP3, has a molecular weight of 3.746×10^6 g/mol. Based on the known molecular weight, the total protein concentration determined by quantitative amino acid analysis of the highly purified AAV empty capsids, and the measured absorbance values, the molar extinction coefficients of capsids at 260 and 280 nm were estimated at 3.72×10^6 and 6.31×10^6 M^{-1} cm^{-1}, respectively. The absorbance of the vector DNA depends on the size of the genome. The molar extinction coefficient at 260 nm for the vector genome ($\epsilon_{260(DNA)}$) was calculated at 20 g^{-1} cm^{-1} $\times$ MW_{DNA} (assuming 50 μg/ml = 1 absorbance unit/cm) and 11.1 g^{-1} cm^{-1} $\times$ MW_{DNA} at 280 nm (assuming an A_{260}/A_{280} ratio of 1.80). The individual absorbance of capsid protein and vector genome can be added to arrive at the extinction coefficient for a vector particle. AAV vector preparations purified by column chromatography may contain a variable amount of empty particles. The extinction coefficient of a mixed population of empty and full vector particles was calculated at each wavelength by adding the extinction coefficients of the DNA and capsids, as expressed in the following equations:

$$\epsilon_{260(\text{vector})} = 20.0 \times \text{MW}_{\text{DNA}} + 3.72 \times 10^6 \times \text{cp/vg}, \quad (1)$$

$$\epsilon_{280(\text{vector})} = 11.1 \times \text{MW}_{\text{DNA}} + 6.31 \times 10^6 \times \text{cp/vg}. \quad (2)$$

The absorbance ratio (A_{260}/A_{280}) is equal to the ratio of the two extinction coefficients:

$$A_{260}/A_{280} = \frac{\epsilon_{260(\text{vector})}}{\epsilon_{280(\text{vector})}} = \frac{20.0 \times \text{MW}_{\text{DNA}} + 3.72 \times 10^6 \times \text{cp/vg}}{11.1 \times \text{MW}_{\text{DNA}} + 6.31 \times 10^6 \times \text{cp/vg}}. \quad (3)$$

The capsid ratio cp/vg can then be expressed as a function of the observed absorbance ratio as follows:

$$\text{cp/vg} = \text{MW}_{\text{DNA}} \times \frac{1.76 \times 10^{-6}(1.80 - A_{260}/A_{280})}{A_{260}/A_{280} - 0.59}. \quad (4)$$

The vector genome concentration (vg/ml) is a function of the absorbance (A_{260}) and the capsid ratio cp/vg, as expressed in the following equation using the extinction coefficient from Eq. (1):

$$\text{vg/ml} = A_{260} \times \frac{6.02 \times 10^{20}}{20 \times \text{MW}_{\text{DNA}} + 3.72 \times 10^6 \times \text{cp/vg}}. \quad (5)$$

Substituting Eq. (4) for cp/vg above allows calculation of the vector genome concentration based solely on the MW of the vector DNA and the observed A_{260} and A_{280}:

$$\text{vg/ml} = \frac{4.47 \times 10^{19}(A_{260} - 0.59A_{280})}{\text{MW}_{\text{DNA}}}. \quad (6)$$

All experiments in this study were done with the recombinant vector AAV-hFIX16. The 4297-nt single-stranded DNA of AAV-hFIX16 has a calculated molecular weight of 1.33×10^6 g/mol, based on the individual MW of the bases in a polymer where A is 312.2 Da, C is 288.2 Da, G is 328.2 Da, and T is 303.2 Da.

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REFERENCES

1. High, K. A. (2001). AAV-mediated gene transfer for hemophilia. *Ann. N. Y. Acad. Sci.* **953:** 64–74.
2. Pfeifer, A., and Verma, I. M. (2001). Gene therapy: promises and problems. *Annu. Rev. Genom. Hum. Genet.* **2:** 177–211.
3. Clark, K. R. (2002). Recent advances in recombinant adeno-associated virus vector production. *Kidney Int.* **61**(Suppl. 1): 9–15.
4. Drittanti, L., *et al.* (2001). Optimised helper virus-free production of high-quality adeno-associated virus vectors. *J. Gene Med.* **3:** 59–71.
5. Gao, G., *et al.* (2000). Purification of recombinant adeno-associated virus vectors by column chromatography and its performance in vivo. *Hum. Gene Ther.* **10:** 2079–2091.
6. Grimm, D., and Kleinschmidt, J. A. (1999). Progress in adeno-associated virus type 2

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vector production: promises and prospects for clinical use. *Hum. Gene Ther.* **10:** 2445–2450.
7. Maizel, J. V. Jr., White, D. O., and Scharff, M. D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* **36:** 115–125.
8. Mittereder, N., March, K. L., and Trapnell, B. C. (1996). Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* **70:** 7498–7509.
9. Sweeney, J. A., and Hennessey, J. P. Jr. (2002). Evaluation of accuracy and precision of adenovirus absorptivity at 260 nm under conditions of complete DNA disruption. *Virology* **295:** 284–288.
10. Lehmberg, E., *et al.* (1999). Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. *J. Chromatogr. B Biomed. Sci. Appl.* **732:** 411–423.
11. Trempe, J. P., and Carter, B. J. (1988). Alternate mRNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. *J. Virol.* **62:** 3356–3363.
12. Xie, Q., *et al.* (2002). The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc. Natl. Acad. Sci. USA* **99:** 10405–10410.
13. Anders, J. (2001). Amino acid analysis. In *BioPharm Guide to Bioanalytical Methods,* supplement to *BioPharm.* Advanstar Communications.
14. Gill, S. C., and von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182:** 319–326 [Erratum published in *Anal. Biochem.,* 1989, **189:** 283].
15. Berns, K. I. (1996). Parvoviridae: the viruses and their replication. In *Fields Virology* (B. N. Fields, D. M. Knipe, P. M. Howley, *et al.*, Eds.), pp. 2173–2197. Lippincott–Raven, Philadelphia.
16. Croyle, M. A., Cheng, X., and Wilson, J. M. (2001). Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Ther.* **8:** 1281–1290.
17. Matsushita, T., *et al.* (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* **7:** 938–945.
18. Qu, G., *et al.* (2001). Characterization of AAV vectors expressing human coagulation factor IX produced at intermediate scale. *Mol. Ther.* **5:** S50.
19. Miao, C. H., *et al.* (2000). Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro. *Mol. Ther.* **1:** 522–532.
20. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. (1996). Real time quantitative PCR. *Genome Res.* **6:** 986–994.
21. Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R., and Kleinschmidt, J. (1999). Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. *Gene Ther.* **6:** 1322–1330.
22. Cantor, C. R., and Schimmel, P. R. (1980). Biophysical chemistry, part II. In *Techniques for the Study of Biological Structure and Function.* Freeman, San Francisco.

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