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Implementing a robust platform analytical procedure for measuring adeno-associated virus vector genome titer

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The vector genome (vg) titer measurement, which is used to control patient dosing and ensure control over drug product manufacturing, is essential for the development of recombinant adeno-associated virus (AAV) gene therapy products. While qPCR and droplet digital PCR technologies are commonly implemented for measuring vg titer, chromatographic techniques with UV detectors represent promising future approaches, in line with traditional biotherapeutics. Here, we introduce a novel vg titer measurement approach using size-exclusion high-performance liquid chromatography with UV detection, which achieves excellent method precision (<2% relative SD), demonstrates linearity across a range of concentrations and varied particle content, is stability indicating, and can be bridged with existing vg titer methods. As there is no bias between this procedure and existing vg titer procedures, such as qPCR, this method can be implemented even at late stages during pharmaceutical development. The procedure was demonstrated to be applicable across serotypes and transgenes, enabling the approach to be used as a platform method for AAV. Given the method performance and criticality of vg titer measurements for AAV, this approach represents a beneficial technology for AAV therapeutics.

INTRODUCTION

Recombinant adeno-associated virus (rAAV) gene therapy vectors consist of small icosahedral capsids containing a single-stranded DNA genome, up to 4.7 kb. The icosahedral capsid comprises a total of 60 capsid proteins, VP1, VP2, and VP3, at an approximately 1:1:10 ratio, although the ratio can vary.[1] While rAAVs may utilize the same capsid sequences and structure, including the ability to leverage different capsid serotypes, rAAVs lack the wild-type AAV protein-coding sequence and replace it with a therapeutic gene expression cassette.[2] AAV vectors represent promising therapeutics due to their positive safety profile, high transduction efficiency, and ability to target various tissues in a specific manner.[3] Approved AAV gene therapy products in the United States and/or European Union include Luxturna, Zolgensma, Hemgenix, Upstaza, Elevidys, Roctavian, and BEQVEZ.

For AAV gene therapy vectors, it is important to consider two concentration measurements, the concentration of the vector genome (vg) and the concentration of the AAV capsid (or vector particle, vp). The vg titer is a measurement of the concentration of vg present in a sample and is expressed in vg/mL, while the vp titer is a measurement of all capsids present, regardless of whether they contain the intended therapeutic transgene. For AAV gene therapy vectors, the vg titer is critically important as it is frequently used for dosing purposes in both the preclinical and clinical spaces.[4,5] Specifically, due to the correlation between gene copy numbers and protein expression, as well as the broader safety considerations for AAV gene therapy products, accurate vg titer measurements are critical to ensure safety and efficacy.[6–8] For this reason, the vg titer is the strength of the gene therapy drug product (DP) and is often included on the DP label.[9]

The initial analytical procedures directed at the quantification of vg titer included dot-blot hybridization, Southern blotting, UV spectrophotometry, and ELISA.[10–13] The introduction of qPCR represented a promising improvement for determining the vg titer and is now commonly implemented, in part due to the high tolerance to matrix impurities, high sensitivity, and specificity.[14–16] A general framework has been developed to standardize critical elements of qPCR and improve the reliability and reproducibility of qPCR results.[17] The quantitation by qPCR can be impacted by primer and probe design, secondary structure in the template, and the presence of inhibitors in the PCR reaction.[4,18,19] Furthermore, qPCR requires a well-characterized standard, most often a linearized plasmid, which requires reagent management, establishing the initial quantitation and reagent bridging if a resupply is needed.[16,20] In addition to qPCR, droplet digital PCR (ddPCR) has been employed for vg titer.[4,5,21] Several studies have reported that ddPCR is more robust and less variable than qPCR, especially for in-process samples.[5,22] Additionally, ddPCR does not require a standard curve.[23,24] The performance of ddPCR has been enhanced by refining the pre-treatment procedure, DNase

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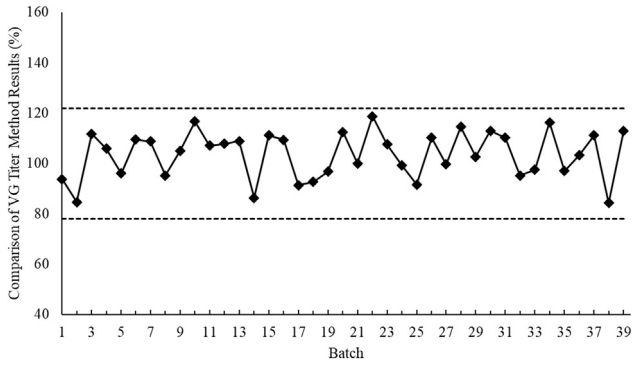


Figure 1. SE-HPLC vg titer values as a percentage of qPCR values

Black diamonds represent the SE-HPLC vg titer as a percentage of the qPCR value (SE-HPLC vg titer/qPCR vg titer × 100) for individual batches of material. Dashed lines represent method variability for the qPCR procedure, which is the target ±3× the SD. The vg titer for drug substance batches and drug product lots used in this evaluation ranged from 8.27E13 to 1.27E14.

digestion, and material handling.[25] The disadvantages of ddPCR are that it has more limited throughput than qPCR and is associated with a higher overall cost, inclusive of instrumentation, consumables, and reagents.[23] Even following significant optimization, the relative SD (RSD) of qPCR and ddPCR can still approach 5% or higher for certain serotypes and constructs.[23,25,26]

While spectrophotometry or UV-based measurements for product concentration are common for traditional biotherapeutics, the techniques have been implemented less frequently for AAV gene therapy products. This is primarily due to the challenge of distinguishing the UV signal from the AAV capsid itself from the DNA packaged inside of the capsid. Additionally, this challenge is further complicated by the heterogeneous nature of AAV products, which can be packaged with the full genome of interest, partially packaged, or not packaged with DNA.[27] Given the criticality of the vg titer measurement, the challenges associated with deriving the vg titer from UV absorbance measurements have limited the application of UV-based procedures, although some publications have been centered around this approach. Optical density measurements were introduced as alternatives to qPCR and ELISA for adenovirus, but required viral disruption and were limited by the impact of impurities and aggregation on the UV absorbance.[28,29] More recently, a size-exclusion high-performance liquid chromatography (SE-HPLC) method was implemented, enabling the separation of AAV capsids from aggregates and impurities prior to the titer measurement.[8] SE-HPLC coupled to multiangle light scattering (MALS) was introduced to monitor and characterize aggregation, size distribution, molecular mass of the capsid and DNA, particle content, and vp and vg titer, and it was considered easy to implement for precise and accurate measurements.[8,27,30] Similar approaches have been developed with alternate chromatographic separations, such as ion-exchange chromatography (IEX).[31] While MALS detectors are suitable for vg titer calculations, UV detectors are also amenable. McIntosh et al. demonstrated the ability to compute vg titers of unknown samples against an AAV standard curve, although the vp titer was underestimated and the vg titer was overestimated as the capsid content changed, specifically as the percentage of "light" capsids, often referred to as empty capsids, increased.[8]

This study describes the implementation of an SE-HPLC UV method for the quantitation of vg titer. Unlike prior studies, this approach does not require the use of a standard curve and demonstrates linearity across a range of empty capsid abundances. The procedure was validated in accordance with International Council for Harmonization (ICH) Q2(R2). When implemented, the procedure can precisely and accurately quantitate the vg titer. The procedure can be applied to multiple serotypes and therapeutic transgenes and can make an immediate impact in patient dosing and aid in biotherapeutic development.

RESULTS

Comparison of qPCR and SE-HPLC vg titer results for an AAV therapeutic

An analytical procedure to compute the vg titer from SE-HPLC UV data was developed and is presented in the materials and methods section. The vg titer values, measured using qPCR and SE-HPLC, were compared across 39 drug substance (DS) batches and DP lots for an AAV9 therapeutic to assess the consistency or accuracy of the SE-HPLC results to those from qPCR. The SE-HPLC vg titer was computed as a percentage of the qPCR vg titer and graphed (Figure 1). SE-HPLC vg titer results averaged 103% of the qPCR vg titer. All SE-HPLC vg titer values fall within ±3 SDs of the qPCR value, as the qPCR procedure has an RSD of 7.3% (Figure 1). Therefore, all vg titer values obtained by SE-HPLC are within the method variability for the qPCR procedure itself. These data indicate no systemic bias exists between the two analytical procedures and support usage of the SE-HPLC procedure for measuring vg titer.

Validation of the SE-HPLC vg titer analytical procedure

The analytical procedure was validated in accordance with ICH Q2(R2) guidelines for precision, accuracy, linearity, specificity, and range. A summary of the validation is presented in Table 1. Method precision and linearity are discussed specifically in subsequent sections here, which include data from the validation and additional supportive data. Based on the validation, the analytical procedure was demonstrated to be a suitable method for measuring the vg titer of AAV.

Method accuracy, or the closeness of the results obtained to their theoretical values, ranged from 92% to 93% accuracy across the range of sample concentrations. As discussed previously with Figure 1, the analytical results are within the variability of the qPCR procedure. While the 92%–93% accuracy observed in this experiment may appear to differ from that observed in Figure 1 (103%), the experimental design should be considered when evaluating these results. Figure 1 was generated by computing individual qPCR and SE-HPLC results from 39 different DS and DP batches, while the data in Table 1 only consider one material injected at different injection levels, so it is not surprising that all accuracy values were similar. Given this design, the qPCR measurement was only performed on

Table 1. Summary of the SE-HPLC vg titer validation

Validation characteristic	Experimental design	Validation results
Method repeatability	3 preparations at 3 levels tested over 1 instance (n = 3 at 3 levels, 3 total preparations); expressed as RSD (%) from the 3 results across each of 3 levels	sample A = 0.3% sample C = 0.0% sample E = 0.3%
Intermediate precision	1 preparation of each sample at 3 levels tested over 6 instances with 2 different analysts on 2 instruments and 2 columns (n = 6 at 3 levels; 6 total preparations); expressed as RSD (%) from the 6 results across each of 3 levels	sample A = 0.6% sample C = 0.5% sample E = 1.5%
Reproducibility	1 preparation of each sample at 3 levels tested over 12 instances in 2 labs with different analysts, instruments, and columns; expressed as RSD (%) from the results across each of 3 levels	sample A = 1.7% sample C = 1.3% sample E = 1.1%
Accuracy	1 preparation of each sample at 3 levels (n = 6); the accuracy (%) for each sample is reported	sample A = 92% sample C = 92% sample E = 93%
Specificity	the response of formulation buffer and blank compared to the response of a tested sample with AAV	response <1% of the sample
Linearity	linearity was evaluated by measuring analytical response of 5 vg titer column loads spanning ∼35%–320% of target column load and by plotting (1) the mean measured genome titer vs. its corresponding theoretical genome titer (2) response factor of each injection level ($P_{DNA\ (SMP)}$) vs. corresponding column load; the plots were analyzed to obtain parameters that were evaluated against the acceptance criteria	(1) appears linear (2) appears linear (1) R^2 = 1.00 (2) R^2 = 1.00
Range	the range of the analytical procedure was determined from an evaluation of the data from linearity, precision, and accuracy experiments	3.99E13–3.65E14 vg/mL

Injection concentrations were as follows: sample A = 3.99E13 vg/mL, sample B = 7.98E13 vg/mL, sample C = 1.14E14 vg/mL, sample D = 1.71E14 vg/mL, and sample E = 3.65E14 vg/mL. The same sample was used for all levels by altering the injection volume. Thus, the calculation to compute vg/mL did not account for injection volume differences between the reference and samples for this exercise. The qPCR concentration was measured for sample C and was calculated for all other samples based on the injection load.
For linearity, vg titer loads were as follows: sample A = 3.99E11 vg, sample B = 7.98E11, sample C = 1.14E12, sample D = 1.71E12, and sample E = 3.65E12 vg. Samples B and D were tested for linearity only.

sample C, and other samples used calculated qPCR vg titer values based on the injection load. As shown in Figure 1, 92%–93% SE-HPLC vg titer as a percentage of the qPCR value (SE-HPLC vg titer/qPCR vg titer × 100) is within the expected range.

Specificity was demonstrated by comparing the UV response of both a formulation buffer and a water blank to that of a sample and demonstrating that the UV response was suitably low. In both cases, the responses were less than 1% of the sample (Table 1), demonstrating that the method is specific to AAV-containing material. Figure 3C further supports the specificity of the method, showing that the method is specific to DNA containing AAV material, as demonstrated by the linear decline in vg titer as the number of full capsids decreases.

Finally, based on the totality of validation data (Table 1) and acceptable levels of linearity, precision, and accuracy, the assay range was determined for this AAV product to be from 3.99E13 to 3.65E14 vg/mL, or injection loads of 3.99E11 vg to 3.65E12 vg. Importantly, this range was based on the needs of this technique for the specific AAV product to quantitate vg titer within the defined allowable ranges for an intermediate step and the final DS and DP material, but it is likely that the method is robust across much broader ranges that were not tested in the method validation exercise. For instance, a linear response was observed for this product in Figure 3C down to concentrations approaching 1E13 vg/mL. In subsequent data for alternate AAV products, the method continued to perform well even below 1E13 vg/mL (Figure 5).

Assessment of method precision

A single assay control sample was analyzed over time to understand the reproducibility of the SE-HPLC analytical procedure. In total, 28 injections were analyzed, with data being acquired by 3 testing sites, 6

Table 2. Summary statistics and VCA for the assay control sample

Summary statistics				Variance component information			
N	Mean	SD (VCA)	RSD, %	Component	Var component	% of total	SD (VCA)
28	9.71E13	1.59E12	1.64	Testing site	1.15E22	0.5	1.07E11
				Analyst (testing site)	3.98E23	15.7	6.31E11
				Replicate	2.13E24	83.8	1.46E12
				Total	2.54E24	100.0	1.59E12

N, sample number; RSD, relative SD; SD (VCA), SD from the variance component analysis (VCA); Var, variance.

analysts, and 14 testing instances to understand the total analytical variability. Summary statistics, including sample size (*N*), mean, SD, and RSD, are provided in Table 2. Variance component analysis (VCA) was applied to estimate the effect of each factor—testing site, analyst and replicate, and the total variance. The VCA results are shown in Table 2. The total SD was then calculated from the square root of the total variance. Figure 2A shows the variability chart broken down by testing site and analyst.

The procedure was determined to be precise and robust across testing locations. The largest source of variability was from within the experiment (denoted as replicate in Table 2) and is associated in general with analytical variability of the procedure, as opposed to a specific source of variability. A small proportion of the variability was attributed to the site-to-site (0.5% of total variance) and analyst (15.7% of total variance) components, demonstrating that the procedure is robust and able to be transferred across analysts and testing sites.

During method validation, the precision of the analytical procedure was evaluated for method repeatability, intermediate precision, and reproducibility (Table 1). Method repeatability was evaluated at 3.99E13, 1.14E14, and 3.65E14 vg/mL, with the highest RSD of any level being 0.3%. These data demonstrate that the method itself is highly precise. Intermediate precision was assessed to understand the precision of the procedure under a variety of intra-laboratory conditions such as multiple instruments, columns, and analysts. These data have a maximum RSD for any level of 1.5%. Finally, reproducibility was evaluated under a variety of inter-laboratory conditions, including multiple laboratories, instruments, columns, and analysts. These data had a maximum RSD of 1.7%. All reproducibility data were graphed and demonstrate suitable precision across a wide range of vg titers, with no systemic bias across testing sites, and acceptable precision at each level (Figures 2B–2D).

Evaluation of method linearity

Method linearity, or the demonstration that there is a linear relationship between analyte concentration and response, was evaluated in several ways during method validation, as summarized in Table 1. Initially, five vg titer loads from 3.99E11 to 3.65E12 vg were injected in the system. The results from three replicates were averaged. Across the entire vg titer range, a linear fit was observed, with an R^2 value of 1.00 (Figure 3A). Similarly, the peak area (PA) at 260 nm attributed to DNA (as defined in Equation 5 in materials and methods) for a given sample also exhibited a linear plot, with an R^2 value of 1.00 across the column load range (Figure 3B).

In addition to the validation data referenced above, an empty AAV capsid spiking study, where empty and full capsids were enriched by cesium chloride (CsCl) ultracentrifugation, was performed to evaluate linearity in the vg titer measurement by varying the vp content between 0% and 100% empty AAV capsid particles (Figure 3C). The samples were generated by spiking in CsCl purified empty and full capsids together at defined ratios, while maintaining a constant vp titer. Across the entire range, the samples had a linear trend (R^2 = 1.00), with the vg titer values decreasing as the empty capsid content increased. Although vg titer values decreased as genome-packaged particle content dropped, the vp titer remained consistent, as expected. These data demonstrate that the procedure accurately calculates vg titer across a range of particle contents and is linear.

Analysis of thermally degraded material

To demonstrate that the SE-HPLC analytical procedure is stability indicating, material was intentionally degraded by storing for up to 2 weeks at 25°C. The material was monitored for vg titer by qPCR and for vg titer and vp titer by SE-HPLC. Importantly, the vg titer by qPCR and SE-HPLC are equivalent (Figure 4A), with both procedures being stability indicating. Additionally, while the vp titer shows some loss, the rate of loss for the vg titer is more pronounced, implying DNA loss or ejection from the capsid itself. After 2 weeks at 25°C, the vg titer was approximately 35% lower, while the vp titer was approximately 15% lower. Rodriguez et al. reported a similar observation, where vg titer is lost at a more profound rate than vg titer at 25°C, although that study showed more consistency in particle titer at 25°C.[32]

To further interrogate the hypothesis that DNA is ejected from the capsid, an additional study was performed, where a sample was stressed for 4 weeks at 25°C. Compared to the unstressed sample (Figure 4B), the AAV vector PA decreased and an earlier-eluting peak (typically evaluated as an aggregate species in SE-HPLC) increased significantly in the stressed sample (Figure 4C). A DNase digestion was performed to digest any DNA that was not encapsidated in the AAV, and the material was then analyzed by SE-HPLC (Figure 4D). This process resulted in the elimination of the earlier-eluting peak,

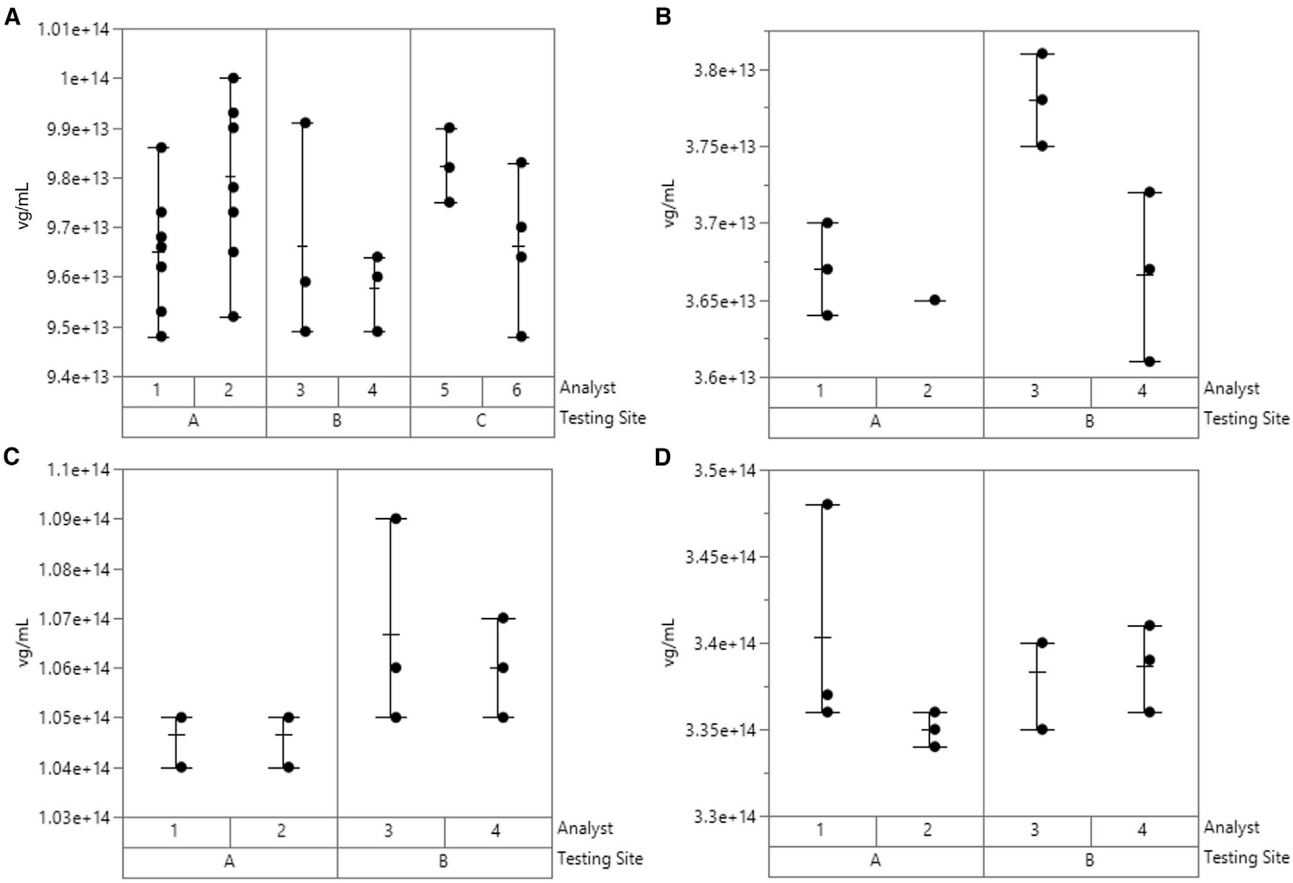


Figure 2. Variability chart for assay control sample

(A) The vg titer (vg/mL) results from six analysts (1–6) located at three testing sites (A–C) for an assay control sample were collected. Each data point is graphed as a black circle. The minimum, maximum, and mean values within an analyst are marked with a horizontal line, respectively. Validation data for samples at 3.99E13 (B), 1.14E14 (C), and 3.65E14 vg/mL (D) are shown. The vg titer results were obtained from four analysts located at two testing sites.

suggesting that the species is related to vector genomic DNA and not aggregated capsids. Meanwhile, the AAV vector peak profile and vg titer for the DNase digested (4.41E13 vg/mL) and undigested (4.39E13 vg/mL) remained consistent. These data demonstrate that the DNase only digests DNA that is not encapsidated within the AAV capsid, and the consistent vg titer values show that the SE-HPLC vg titer procedure is specific to DNA packaged inside the capsid.

SE-HPLC vg titer procedure is a platform method

While the SE-HPLC vg titer analytical procedure was successfully implemented for one AAV product, two additional AAV products were evaluated, totaling an assessment of three distinct AAV capsid serotypes and three distinct transgenes. Furthermore, each AAV product used diverse manufacturing processes, where two AAV products were manufactured with a commonly implemented HEK293 triple transfection processes and one leveraged *Spodoptera frugiperda* (Sf9) and recombinant baculovirus (rBV) technology.[33–35] The downstream purifications also varied between AAV products.

As with the linearity study referenced above, an empty capsid spiking study was used to demonstrate linearity in vg titer measurements for samples of variable particle content levels, ranging from a target of 0% full capsids to 100% full capsids (Figure 5). The samples were generated by spiking in CsCl-purified empty and full capsids together at defined ratios, while maintaining a constant vp titer. The 100% full samples were measured by SE-HPLC and qPCR for the vg titer, both measurements giving similar titer values for the AAVx (Figure 5A, inset) and AAV6 (Figure 5B, inset) products. Additionally, the measured vg/mL value for both products shows a linear trend that approaches 0 vg/mL for the 0% full capsid material. These data demonstrate that the SE-HPLC vg titer procedure is applicable to additional AAV serotypes and therapeutic-specific transgenes.

Impact of improved precision on manufacturing and batch failure

As with all biotherapeutics, AAV vector batch quality is controlled in part by the specification acceptance criteria established for each quality attribute tested at batch release.[36] These acceptance criteria ensure

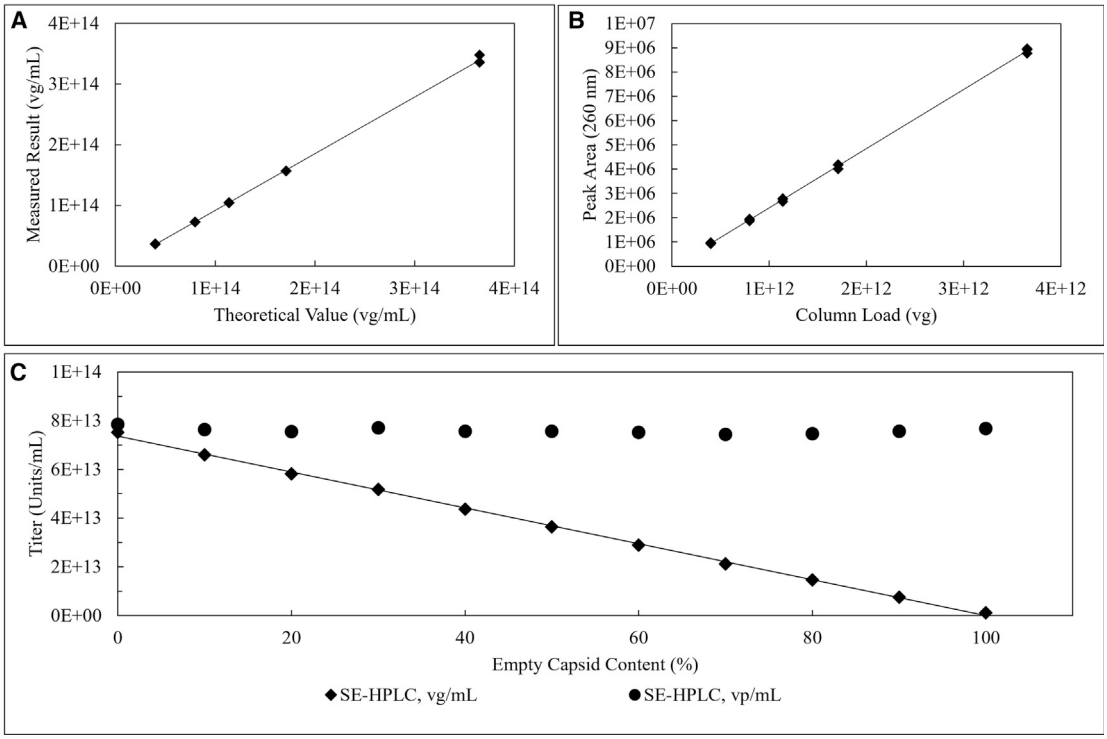


Figure 3. Linearity of the SE-HPLC analytical procedure

(A) The vg titer was computed for various injection loads to mirror a range of vg/mL concentrations. Black diamonds represent individual data points (n = 3 at each theoretical level), with the solid line representing the trend line. (B) Peak area (PA) at 260 nm attributed to DNA was computed for various injection loads, omitting the impact of injection volume from the vg/mL calculation. Black diamonds represent individual data points (n = 3 results at each load level), with the solid line representing the trend line. Black diamonds represent individual data points (n = 3 results at each load level), with the solid line representing the trend line. (C) CsCl purified empty and packaged AAV samples were mixed at various levels, and vg titer and vp titers were measured. The solid line represents the trend line for vg titer vs. empty capsid content.

consistent batch-to-batch quality, as well as safety and efficacy. In the case of vg titer, acceptance criteria can also be used to ensure consistent product dosing. The ability to meet an acceptance criterion is impacted by the manufacturing process performance, the analytical variability, and the acceptance criterion range. To demonstrate the importance of analytical variability on meeting the established acceptance criterion, a statistical simulation was used to estimate the batch failure rate for an AAV vector DP using a representative manufacturing process. Briefly, the manufacturing includes overconcentrating the vg titer at an intermediate step, called the DS sublot, measuring the vg titer at that step, and using the measurement to direct the dilution to the target vg titer at DS. Following the DS vg titer measurement, DS was refiltered and vialed into DP, where the final DP release testing occurs.

The simulation was computed across assay precision values, from 1% to 15% RSD, and batch acceptance criteria ranged from 70% to 130% of the target, down to 99%–101% of the target vg titer. A tabulated view of all failure rates across the acceptance criteria range and analytical procedure RSD values is provided in Figure 6A, which is color-coded to indicate the probability of an analytical result being outside of the specification acceptance criterion. As expected, there is an inverse correlation for the rate of batch failure between the width of the acceptance criterion and the analytical procedure RSD. For a given batch, a wide batch release acceptance criterion and a small analytical procedure RSD (i.e., high precision) have a high probability of batches consistently meeting the acceptance criterion, whereas a narrow batch release acceptance criterion and a high analytical procedure RSD would result in a high number of batch failures (and risk failing batches of otherwise acceptable quality) (Figure 6A). The batch failure rate is impacted by two predominant factors: the variability of the analytical procedure, as discussed below, and the impact that the in-process measurement has on the process performance. As the simulation assumes the dilution is based on an in-process measurement, the implementation of a procedure with a higher RSD results in poorer accuracy of the dilution and more results that are outside of the acceptance criterion.

In addition to minimizing the frequency of batches outside of the acceptance criterion, the improvement in analytical procedure precision has additional benefits. As a statistical simulation was performed, both the measured and actual vg titer values are known, allowing the computation of type I and type II errors associated with the measurements. In this case, a type I error represents the failure of a "good batch," denoted by an analytical result that measures outside of the acceptance criterion (out of the specification test result), but the

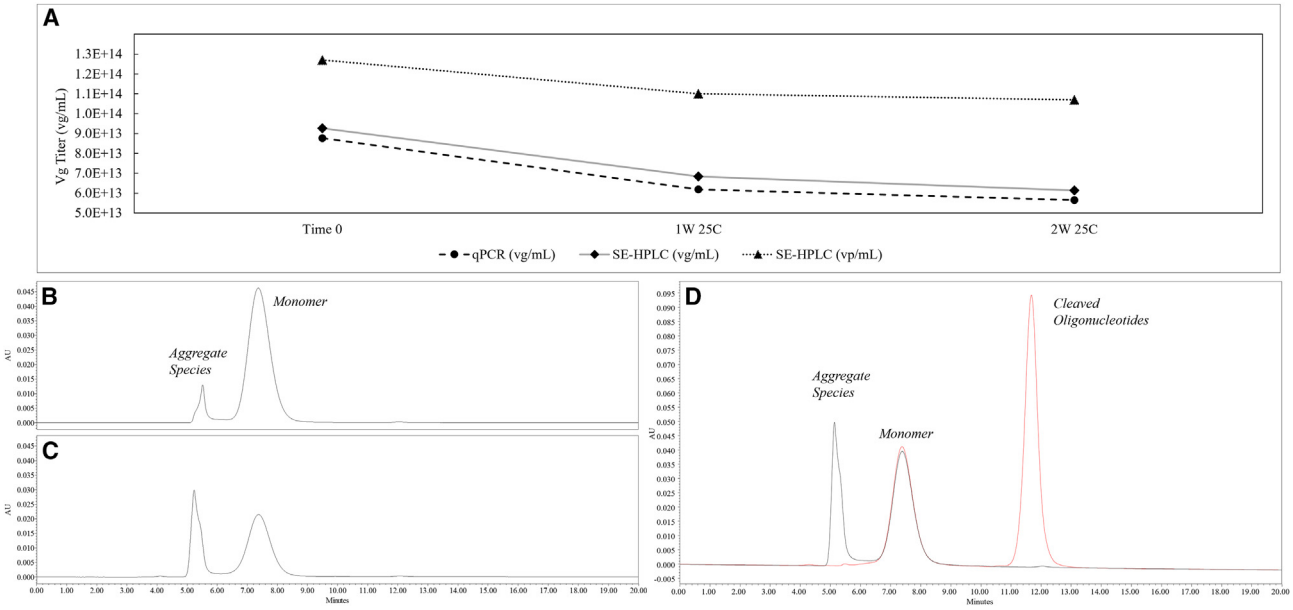


Figure 4. SE-HPLC is a stability-indicating procedure

(A) The vg/mL values computed by qPCR and vg/mL and vp/mL values computed by SE-HPLC over 2 weeks at 25°C. (B) The UV profile at 260 nm of the unstressed sample. (C) A separate sample was stressed for 4 weeks and was reassessed. (D) The 4-week stressed sample was analyzed with (red) and without (black) DNase.

true value is within the acceptance criterion. Practically speaking, the failure of a good batch results in less inventory available for patients and higher manufacturing costs. A type II error represents the acceptance of a "bad batch," denoted by an analytical result that is measured to be within the specification acceptance criterion, but the true value is outside of the acceptance criterion. Passing a batch where the true value does not meet the vg titer criterion means that patients receive a different amount of material than expected and could impact the safety or efficacy of the product. Figure 6B depicts the type I and type II errors based on acceptance criterion range and the analytical procedure precision.

To illustrate the impact of moving from qPCR (RSD of 7.3%) to SE-HPLC (precision of 1.7% based on reproducibility data), batch failure rates, type I, and type II errors were evaluated at nominal percentages closest to the individual procedure percent RSDs. When looking at an example where the acceptance criterion is the target ±10%, the total batch failure rate based on the model was 1.2% when the analytical procedure RSD was 2% and 32.6% when the analytical procedure RSD was 7% (Figure 6A). Likewise, the probability of type I error decreased from 20.7% to 1.0% and the probability of type II error decreased from 6.1% to 0.2% when comparing analytical procedure precision of 7% vs. 2% RSD (Figure 6B). Beyond an acceptance criterion of 10%, the frequency of batch failure, type I error, and type II error decreases dramatically when the analytical procedure RSD was 2%. These data illustrate the criticality of minimizing analytical procedure variability for the vg titer procedure, because doing so reduces the total number of batch failures, the probability of discarding acceptable batches, and the probability of accepting failed batches.

DISCUSSION

A novel SE-HPLC method to measure the vg titer of an AAV therapeutic was developed and demonstrated as a viable alternative to qPCR-based methodologies. The performance was evaluated to ensure alignment with the requirements of analytical procedures for concentration per ICH Q2(R2). The analytical procedure is specific, precise (Figure 2; Table 2), has a linear response (Figure 3), has an established range (Table 1), is robust (Table 2), and is stability indicating (Figure 4). Additionally, and critical to establishing the SE-HPLC procedure for AAV therapeutics already in the clinic, the procedure can be successfully bridged to an existing vg titer measurement (i.e., by qPCR) through the use of a reference material (RM), resulting in no bias between the analytical procedures and demonstrating accuracy to an established method (Figure 1). The procedure can also be directly applied to multiple AAV serotypes, transgenes, and manufacturing parameters (Figure 5). While this publication demonstrates that the procedure is precise and robust, there are several additional considerations for implementing an SE-HPLC-derived concentration of vg titer.

The SE-HPLC vg titer procedure described here can be successfully implemented as part of the overall control strategy for gene therapy vector development. While a major advantage of ddPCR or qPCR is the specificity achieved via the use of product-specific primers that target the therapeutic transgene, this is not a requirement of the SE-HPLC vg titer analytical procedure, as additional analytics in the broader analytical control strategy are in place to ensure genome identity and integrity.[37–41] The SE-HPLC vg titer procedure was shown to be specific for DNA packaged inside of the capsid and

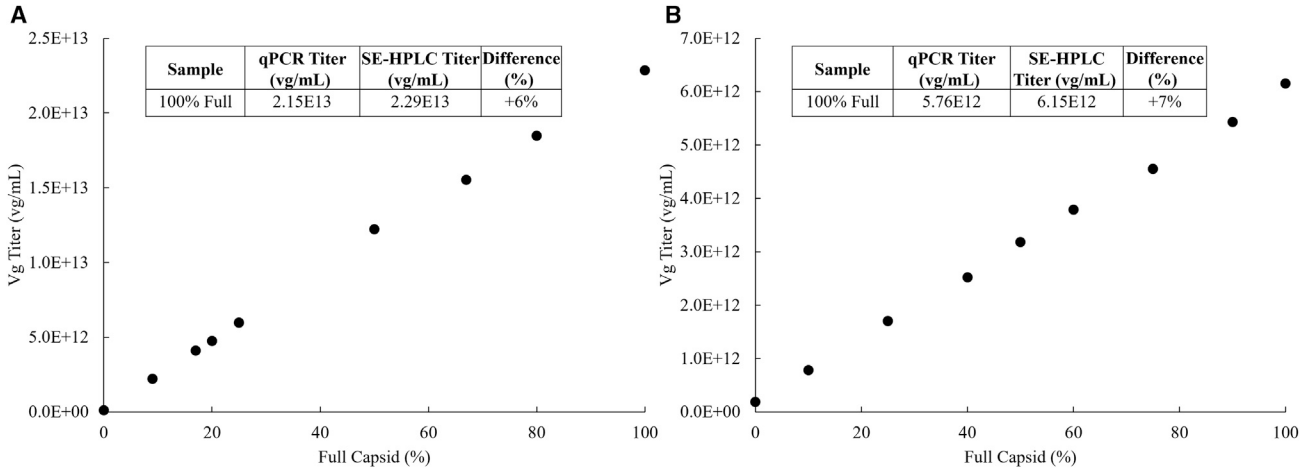


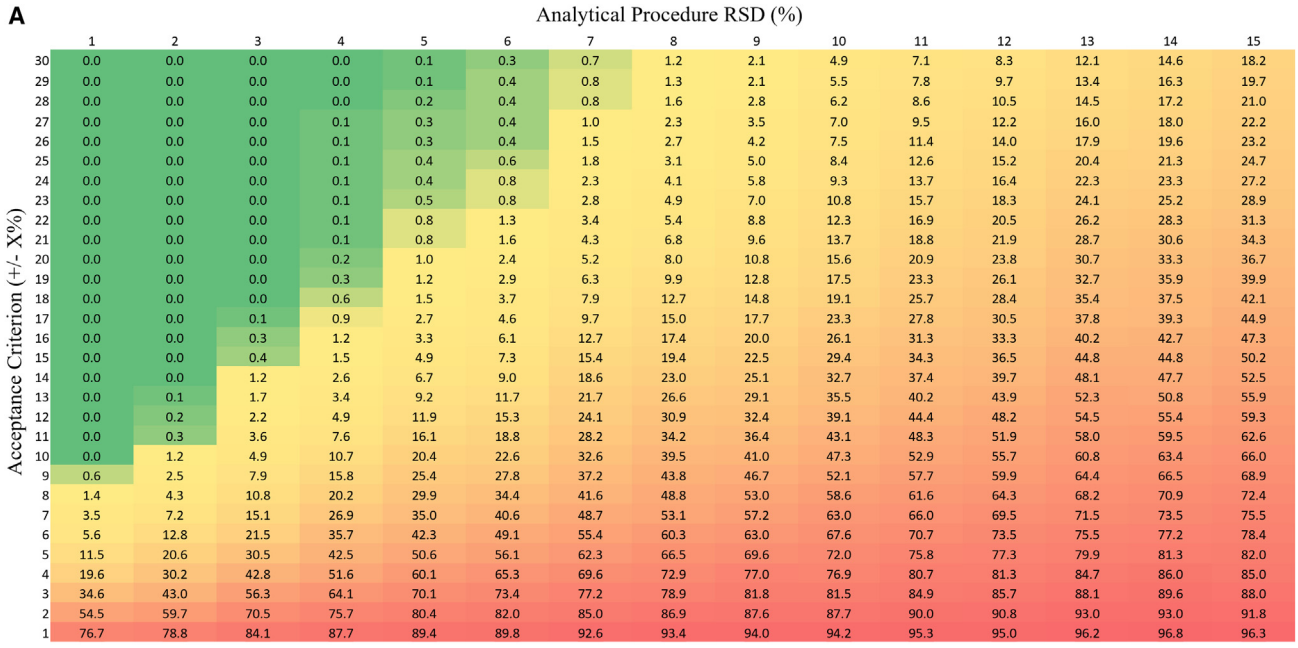
Figure 5. SE-HPLC vg titer measurements for alternate serotypes and transgenes

(A) The vg/mL values computed by SE-HPLC for an AAVx product. (B) The vg/mL values computed by SE-HPLC for an AAV6 product. Enriched empty and full capsids were prepared via cesium chloride ultracentrifugation and spiked at defined levels. The inset shows a comparison of the qPCR and SE-HPLC vg titer values for the 100% full capsid by qPCR.

measured DNA levels in AAV samples with far greater precision than PCR-based methods. Residual DNA impurities (i.e., host cell DNA, BV DNA, and residual plasmid) that could interfere with or bias the SE-HPLC vg titer procedure are removed to low levels through the downstream manufacturing process, and the control strategy includes release testing to ensure that these are at acceptable levels.[37] Any remaining DNA impurity would have a limited vg titer impact and only in the case where these residual impurities co-elute or are co-packaged with the AAV vector by SE-HPLC. For the study here, very low levels of residual host cell DNA and plasmid were observed in the product. Additionally, assuming a robust manufacturing process with control of residual impurities, the SE-HPLC measurement is made relative to the vg titer of the RM, which can be established from a method that targets the transgene (i.e., qPCR or ddPCR) and compensates for the impact of these residual impurities. It is recommended that the vg titer of the RM be established by replicate testing to ensure confidence in the RM vg titer value and account for any variability in the test used to establish the value. While there may be perceived disadvantages about not using a product-specific primer, this feature could also be considered an advantage of the SE-HPLC procedure, as it enables a platform method that can be applied across all gene therapy programs, assuming an applicable RM, instead of requiring a product-specific method and reagents. As with residual impurities, the SE-HPLC procedure can quantitate the vg titer for products with variable levels of particle content or the ratio of capsids packaged with the full genome of interest, partially packaged or not packaged with DNA. One particular challenge is partially packaged capsids, which have DNA and would therefore be detectable by the SE-HPLC procedure. A robust manufacturing process should ensure consistency in particle content, including partially packaged capsids, in which case the inclusion of the RM will compensate for the impact of partially packaged capsids on the final vg titer reportable value. Additional release procedures are included in the analytical control strategy to ensure that the particle content remains consistent.[27]

Another advantage to the SE-HPLC vg titer procedure is ease of implementation to a new program or a program with an extensive manufacturing change resulting in a change in the product; however, some challenges warrant discussion. When applied to a new program early in development, a product-specific RM may not be available. This can be mitigated by establishing a program-specific qPCR/ddPCR-based method, which would likely be required and/or already developed for upstream sample testing. Upstream or in-process samples are likely incompatible with the SE-HPLC vg titer procedure due to complex matrices and lower product titers; however, a less precise qPCR/ddPCR-based method is acceptable for testing these samples. Thus, a comprehensive control strategy could implement qPCR/ddPCR for upstream or early in-process samples and transition to SE-HPLC for vg titer for later in-process samples or final DS and DP samples. In this case, connectivity between vg titer measurements is best ensured by establishing the RM vg titer with the same qPCR/ddPCR method used for in-process testing. Alternatively, a RM from a different program, ideally with characteristics similar to those of the new program, such as capsid serotype and genome size, could be used as the RM. While the SE-HPLC procedure is robust across particle content ranges, differences in extinction coefficients for the capsid and genome, the abundance of partially packaged species, or the level of residual impurities could result in bias between products. Any differences would have to be evaluated for acceptability. It should be noted that establishing a baseline vg titer value of a RM by qPCR poses challenges, as the value can be impacted by the use of linearized vs. supercoiled plasmid standards, the decision to pre-treat with Proteinase K, and the specific amplicon targeted in the procedure.[19,20] A new RM, or the application of a correction factor, may be required for process changes that result in significant product differences that

A



Acceptance Criterion (+/- X%)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
30	0.0	0.0	0.0	0.0	0.1	0.3	0.7	1.2	2.1	4.9	7.1	8.3	12.1	14.6	18.2
29	0.0	0.0	0.0	0.0	0.1	0.4	0.8	1.3	2.1	5.5	7.8	9.7	13.4	16.3	19.7
28	0.0	0.0	0.0	0.0	0.2	0.4	0.8	1.6	2.8	6.2	8.6	10.5	14.5	17.2	21.0
27	0.0	0.0	0.0	0.1	0.3	0.4	1.0	2.3	3.5	7.0	9.5	12.2	16.0	18.0	22.2
26	0.0	0.0	0.0	0.1	0.3	0.4	1.5	2.7	4.2	7.5	11.4	14.0	17.9	19.6	23.2
25	0.0	0.0	0.0	0.1	0.4	0.6	1.8	3.1	5.0	8.4	12.6	15.2	20.4	21.3	24.7
24	0.0	0.0	0.0	0.1	0.4	0.8	2.3	4.1	5.8	9.3	13.7	16.4	22.3	23.3	27.2
23	0.0	0.0	0.0	0.1	0.5	0.8	2.8	4.9	7.0	10.8	15.7	18.3	24.1	25.2	28.9
22	0.0	0.0	0.0	0.1	0.8	1.3	3.4	5.4	8.8	12.3	16.9	20.5	26.2	28.3	31.3
21	0.0	0.0	0.0	0.1	0.8	1.6	4.3	6.8	9.6	13.7	18.8	21.9	28.7	30.6	34.3
20	0.0	0.0	0.0	0.2	1.0	2.4	5.2	8.0	10.8	15.6	20.9	23.8	30.7	33.3	36.7
19	0.0	0.0	0.0	0.3	1.2	2.9	6.3	9.9	12.8	17.5	23.3	26.1	32.7	35.9	39.9
18	0.0	0.0	0.0	0.6	1.5	3.7	7.9	12.7	14.8	19.1	25.7	28.4	35.4	37.5	42.1
17	0.0	0.0	0.1	0.9	2.7	4.6	9.7	15.0	17.7	23.3	27.8	30.5	37.8	39.3	44.9
16	0.0	0.0	0.3	1.2	3.3	6.1	12.7	17.4	20.0	26.1	31.3	33.3	40.2	42.7	47.3
15	0.0	0.0	0.4	1.5	4.9	7.3	15.4	19.4	22.5	29.4	34.3	36.5	44.8	44.8	50.2
14	0.0	0.0	1.2	2.6	6.7	9.0	18.6	23.0	25.1	32.7	37.4	39.7	48.1	47.7	52.5
13	0.0	0.1	1.7	3.4	9.2	11.7	21.7	26.6	29.1	35.5	40.2	43.9	52.3	50.8	55.9
12	0.0	0.2	2.2	4.9	11.9	15.3	24.1	30.9	32.4	39.1	44.4	48.2	54.5	55.4	59.3
11	0.0	0.3	3.6	7.6	16.1	18.8	28.2	34.2	36.4	43.1	48.3	51.9	58.0	59.5	62.6
10	0.0	1.2	4.9	10.7	20.4	22.6	32.6	39.5	41.0	47.3	52.9	55.7	60.8	63.4	66.0
9	0.6	2.5	7.9	15.8	25.4	27.8	37.2	43.8	46.7	52.1	57.7	59.9	64.4	66.5	68.9
8	1.4	4.3	10.8	20.2	29.9	34.4	41.6	48.8	53.0	58.6	61.6	64.3	68.2	70.9	72.4
7	3.5	7.2	15.1	26.9	35.0	40.6	48.7	53.1	57.2	63.0	66.0	69.5	71.5	73.5	75.5
6	5.6	12.8	21.5	35.7	42.3	49.1	55.4	60.3	63.0	67.6	70.7	73.5	75.5	77.2	78.4
5	11.5	20.6	30.5	42.5	50.6	56.1	62.3	66.5	69.6	72.0	75.8	77.3	79.9	81.3	82.0
4	19.6	30.2	42.8	51.6	60.1	65.3	69.6	72.9	77.0	76.9	80.7	81.3	84.7	86.0	85.0
3	34.6	43.0	56.3	64.1	70.1	73.4	77.2	78.9	81.8	81.5	84.9	85.7	88.1	89.6	88.0
2	54.5	59.7	70.5	75.7	80.4	82.0	85.0	86.9	87.6	87.7	90.0	90.8	93.0	93.0	91.8
1	76.7	78.8	84.1	87.7	89.4	89.8	92.6	93.4	94.0	94.2	95.3	95.0	96.2	96.8	96.3

B

Procedure RSD	+/- 5% Acceptance Criterion		+/- 10% Acceptance Criterion		+/- 15% Acceptance Criterion		+/-20% Acceptance Criterion	
	Type 1 Errors (%)	Type 2 Errors (%)	Type 1 Errors (%)	Type 2 Errors (%)	Type 1 Errors (%)	Type 2 Errors (%)	Type 1 Errors (%)	Type 2 Errors (%)
2% RSD	9.4	3.5	1.0	0.2	0.0	0.0	0.0	0.0
7% RSD	24.2	13.1	20.7	6.1	11.8	1.5	4.5	0.6

Figure 6. Impact of procedure precision and acceptance criterion on batch failure rates

(A) Tabulated view of the batch failure rate as a function of the analytical procedure RSD and acceptance criteria. (B) Type I and type II errors across acceptance criteria based on the analytical procedure RSD.

would impact the vg titer calculation. Knowledge of product differences can be obtained through routine release testing or characterization testing, including analytical ultracentrifugation and next-generation sequencing, for components like residual impurities or particle content.

Despite the challenges associated with the SE-HPLC vg titer approach, the novel method demonstrates a significant improvement in performance, which may be required for robust and successful commercial manufacturing. For biotherapeutic manufacturing, it is important to maintain adequate control of the process and eliminate batches that do not meet the release testing acceptance criteria, thereby ensuring adequate commercial supply and product quality and minimizing the cost of goods. For a defined process, the ability to manufacture and release a biotherapeutic within the allowable acceptance criterion is impacted by the manufacturing process performance, the analytical variability and the allowable acceptance criterion. As demonstrated in Figure 6A, analytical procedures with better precision are significantly less likely to result in an out-of-specification result due to analytical variability and minimize the frequency of type I and II errors (Figure 6B). This is also important when considering stability testing, which often uses the same analytical procedure and acceptance criterion but requires testing over the entire product period of use. Based on the improvement in method performance over conventional analytical techniques for measuring vg titer, alignment in overall titer values, and robustness and transferability of the analytical procedure, the novel SE-HPLC analytical procedure developed here can be implemented for early- and/or late-stage AAV development and represents an improvement compared to traditional approaches.

MATERIALS AND METHODS

Vector production, purification, and preparation for analysis

AAV material

The AAV materials used in this study are as follows. All materials used in the study are recombinant AAV (rAAV) and are referred to as AAV for simplicity. All therapeutic genomes are flanked by AAV2 inverted terminal repeats.

An AAV9 AAV encoding a proprietary protein sequence was produced through triple transfection of HEK293 cells. Purification involved filtration, affinity chromatography, and IEX chromatography. All manuscript data herein use this AAV product unless otherwise specified.

AAVx represents material that was produced through a triple transfection of adherent HEK293 cells, as described previously.[27] AAVx represents an engineered vector capsid with a proprietary protein sequence. Purification involved filtration and IEX chromatography.

AAV6 represents material that was produced using the insect cell line Sf9 and rBV technology. Purification involved affinity chromatography, filtration, and IEX chromatography.

Empty/full sample preparation

The preparation of enriched empty and full capsids via CsCl ultracentrifugation has been described previously.[27] Briefly, purified capsids are concentrated, mixed with CsCl, and centrifuged at 40,000 rpm to generate distinct high- and low-density viral bands, corresponding to full and empty capsids, respectively. The enriched fractions are isolated via side-puncture, buffer exchanged to remove residual CsCl, and then combined at various ratios, resulting in test samples that span a range of particle content. Prepared spike ratio samples were tested using the vg titer by SE-HPLC and qPCR and using the vp titer by SE-HPLC, as described below.

Analytical procedures

SE-HPLC procedure for vg titer

Capsids were separated from impurities and free DNA by SE-HPLC. The test samples were injected neat, or non-diluted, onto a TOSOH TSKgel column and separated by isocratic elution using a phosphate-buffered mobile phase (pH 7.2). To compute the vg titer (vg/mL), the detection system comprised an HPLC system with a UV-visible (UV-vis) diode array detector collecting at UV wavelengths of 260 and 280 nm as described below. A majority of injections were computed at 10-μL sample injection volumes, but alternate injection volumes can be facilitated, as noted in Table 1 and accounted for in Equation 6.

The DNA content of the capsid can be measured by UV detection at 260 and 280 nm simultaneously. DNA and proteins both absorb at wavelengths of 260 and 280 nm and have defined, sequence-dependent absorption coefficients at both 260 and 280 nm. As a result, the DNA content in a sample can be measured by its UV absorbance at 260 nm. However, in AAV gene therapy product, the UV signal at 260 nm will have contributions from the capsid proteins as well, thus requiring additional information to use UV absorbance at 260 nm to measure DNA content in an AAV capsid. Using the measured absorbances at both 260 and 280 nm in conjunction with the unique extinction coefficients for the specific DNA sequence and the AAV capsid, it is possible to isolate the DNA contribution to a given UV signal in an AAV product, as described below.

For a given DNA or protein molecule, the ratio of its absorbances at 260 and 280 nm is a constant. R_D and R_P are the ratio of responses (PA for SE-HPLC) at 260 and 280 nm for a DNA molecule and a protein molecule, respectively, as detailed in Equations 1 and 2:

$$R_D = PA_{D260}/PA_{D280} \quad \text{(Equation 1)}$$

$$R_P = PA_{P260}/PA_{P280}, \quad \text{(Equation 2)}$$

where PA_{D260} and PA_{D280} are PAs at 260 and 280 nm contributed by the DNA of interest, and PA_{P260} and PA_{P280} are PAs at 260 and 280 nm contributed by the AAV proteins.

Each of the PAs at 260 and 280 nm for the AAV peak is made of two components, contributions from DNA, and protein as expressed in Equations 3 and 4:

$$PA_{260} = PA_{D260} + PA_{P260} \quad \text{(Equation 3)}$$

$$PA_{280} = PA_{D280} + PA_{P280}, \quad \text{(Equation 4)}$$

where PA_{260} and PA_{280} are PAs at 260 and 280 nm measured experimentally, PA_{D260} and PA_{D280} are PAs contributed by the DNA at 260 and 280 nm, and PA_{P260} and PA_{P280} are PAs contributed by the AAV proteins at 260 and 280 nm.

Solving Equations 1, 2, 3, and 4 for PA_{D260} produces Equation 5:

$$PA_{D260} = (PA_{260} - R_P \times PA_{280}) \times R_D \; / \; (R_D - R_P) \quad \text{(Equation 5)}$$

While PA_{260} and PA_{280} are experimentally measured, as stated above, R_D and R_P are constants. In this case, $R_D = 1.64$ and $R_P = 0.57$, which were computed using an *in silico* tool based on commonly accepted extinction coefficients.[42] The calculated absorption coefficients for genome-containing particles used a single-stranded DNA and did not account for the potential hypochromism of bases due to stacking and hydrogen bonding. These values were experimentally confirmed by SE-HPLC of both an enriched full capsid and free DNA and were determined to be acceptable during method development.

Once PA_{D260} is computed for a given sample, the same SE-HPLC analysis is performed for a RM) of the product of interest. After the DNA portion of the PA at 260 nm is calculated for both the sample and the RM using Equation 5, the vg titer for the sample can subsequently be calculated using Equation 6:

$$\text{vg titer (sample)} = \left(\left(PA_{D260\ (\text{sample})} \times V_{RM}\right) \; / \; \left(PA_{D260(RM)} \times V_{\text{sample}}\right)\right) \times \text{vg titer (RM)}, \quad \text{(Equation 6)}$$

where vg titer (sample) and vg titer (RM) are vg titer values of the sample and RM, respectively, $PA_{D260\ (\text{sample})}$ and $PA_{D260\ (RM)}$ are DNA PA at 260 nm of the sample and RM, respectively, and V_{sample} and V_{RM} are injection volume of the sample and the RM, respectively.

SE-HPLC procedure for VP titer, aggregate species, and monomer

As described previously, the test samples were injected neat onto a TOSOH TSKgel column and separated by isocratic elution using a

phosphate-buffered mobile phase.[27] The detection system comprised an HPLC system with a UV-vis diode array detector collecting at UV wavelengths of 214, 260, and 280 nm. The capsid titer (vp/mL) values of the samples were determined using relative PA quantitation at 214 nm against a calibration curve prepared with an AAV standard of a predetermined concentration. SE separates components by hydrodynamic volume, resulting in the elution of AAV capsids at a consistent retention time, regardless of the packaged content. This technique was also utilized to assess the capsid content through a 260/280-PA ratio calculation. The chromatographic peak corresponding to the AAV monomer was integrated at 260 and 280 nm, and the PA ratio (SE A260/A280 ratio) was calculated for each sample. To compute the monomer and aggregate species, the PA at 214 nm corresponding to percentage monomer and percentage aggregate species, respectively, were divided by the total PA at 214 nm and converted to a percentage.

qPCR

AAV test samples were analyzed for genome titer using qPCR technology. Non-encapsidated DNA was digested by treatment with DNase I (AAV6, New England Biolabs; AAV9, MP Biomedicals; AAVx, Invitrogen) at 37°C for 60 min (AAV6), room temperature for 60 min (AAV9), or room temperature for 15 min (AAVx). Digested AAV9 and AAVx samples were treated with detergent/EDTA/NaCl solution, then heated to inactivate the DNase and denature the vector capsid. Digested AAV6 samples were heated to inactivate the DNase, then treated with Proteinase K at 55°C for 30 min to digest the vector capsid, followed by an additional heat treatment to inactivate the Proteinase K. Test samples were then diluted into the assay range for analysis. A standard curve was prepared by serial dilution of linearized plasmid containing the transgene with a known copy number. After addition to the reaction plate, qPCR Master Mix (Applied Biosystems) containing target-specific primers and a TaqMan probe were added to each well. Samples were analyzed on an Applied Biosystems 7500 real-time or fast real-time PCR system, and the concentration of the target sequence was interpolated from the standard curve and subsequently converted to vg/mL.

Statistical methods

VCA

The JMP Quality and Process, Variability/Attribute gauge chart function was used for VCA)and variability plotting for site and analyst effects. The JMP Summary function was used to calculate the overall mean. SD was calculated from the square root of the total variance of VCA.

Statistical analysis of batch failure

A statistical simulation was generated by computing 1,000 data points under defined manufacturing conditions to estimate the batch failure rate as a factor of the acceptance criteria and method precision. For the statistical simulation, a process was construed where DS sublots were manufactured to an overconcentrated vg titer, the vg titer was measured and used to dilute to DS, and the product was then refiltered and vialed to DP. For the simulation, the process was assumed to allow up to 2% process variability for the DS sublot overconcentration, DS dilution, and DP refiltration and vialing. No systemic vg titer differences were assumed moving from DS to DP. Modeled results were computed across analytical precision ranges (RSD 1%–15%) and acceptance criteria ranges (±1%–30%).

DATA AND CODE AVAILABILITY

All data necessary to interpret, verify, and extend the research in the article have been made available.

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AUTHOR CONTRIBUTIONS

T.W.P., C.D.K.S., D.S., S.M., and J.J.M. generated materials, authored protocols, performed experiments, and analyzed data. T.W.P. wrote the manuscript. B.E., K.L., and T.W.P. performed the statistical assessments of the data. T.W.P., S.M., K.L., and D.S. generated figures and tables for the manuscript. All authors were involved in the planning, review, editing, and approval of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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