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A qPCR Method for AAV Genome Titer with ddPCR-Level of Accuracy and Precision

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Recombinant adeno-associated virus (rAAV) is one of the main vectors used in gene therapy. An accurate genome titer is not only critical for clinical dosing, but also a prerequisite for many analytical assays for AAV product characterization. AAV genome titer is traditionally determined by qPCR; however, assay precision is not optimal despite extensive efforts. More recently, droplet digital PCR (ddPCR) emerged as a powerful alternative that offers excellent accuracy and precision. However, currently ddPCR is not as widely available as qPCR and operates at a lower throughput and a higher cost. In this paper, we introduce an improved qPCR method with two major optimizations: (1) using an AAV reference material as qPCR standard instead of plasmid DNA and (2) implementing a "digestion-free" method by adding 5% Tween 20 to standard and sample preparations. The new method has been extensively tested with AAV of different serotypes, purification status, and transgenes encapsidated and was found to be highly accurate, precise, and robust. This significantly improved and simplified assay can be easily adopted by researchers in the gene therapy field and further automated for high-throughput applications.

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is a non-enveloped virus that contains a single stranded DNA genome encapsidated by an icosahedral shell.[1] The quantity of rAAV is usually determined by measuring either the capsid protein or the AAV genome. Due to the existence of empty capsids that do not possess therapeutic benefits, titration by viral genome is preferred for clinical dosing, manufacturing, and analytical testing.[2–4] Traditionally, AAV genome titer is determined by qPCR using a plasmid DNA standard. For the past two decades, extensive efforts have been made in improving qPCR standard preparation, sample treatment, and primer/probe design, etc.[5–7] However, the qPCR method remains variable, and the relative standard deviation (RSD) among different labs could reach over 70%.[8] Currently, the field is quickly moving toward droplet digital PCR (ddPCR), a new PCR technology that can achieve superior accuracy and precision, without the need of standard curves or special sample preparation.[2,9] However, ddPCR has its own shortcomings. Different from qPCR, ddPCR cannot be scaled up from 96 to 384 wells, and each well must be read individually, which limits throughput and prolongs assay time. Moreover, the cost of instruments, consumables and reagents are significantly higher than qPCR.[10] Therefore, an improved qPCR method with better accuracy and precision is highly desirable, especially for high throughput applications and for researchers who do not have access to ddPCR.

The relative potency (RP) method is an effective approach to reducing assay variation and is widely used in cell-based assays, which are among the most variable bioassays.[11] It requires a reference standard run side by side with testing samples, and the choice of reference must ensure biological similarity to samples, so that they can behave like a concentration or dilution of each other. The testing results are reported as % RP of standard, which is defined as 100%.[11] Therefore, even though the raw signals from different runs may vary significantly, the % RP is expected to stay constant. In contrast, the existing qPCR methods use plasmid DNA as standard, which shares little similarity with AAV samples. Conceivably, if standard and samples respond differently to testing variables such as analysts, reagents, and instruments, assay precision will be affected. Moreover, the need of long-term maintenance of a DNA standard adds to the challenge.[12,13] Therefore, we propose using a representative AAV reference material as qPCR standard, so sample genome titers can be calculated relative to the reference titer, which can be pre-determined by ddPCR or the existing qPCR methods. Moreover, we eliminated unnecessary sample treatments by employing a digestion-free method that has been demonstrated to improve qPCR quantification of residual DNA in AAV.[14] As a result, a simplified and more streamlined qPCR method for AAV genome titer has been developed, which is able to achieve ddPCR-level of accuracy and precision.

RESULTS

Establishing an Optimized qPCR Method for AAV Genome Titer

Two potential improvements were tested in this study: (1) using an AAV reference material instead of plasmid DNA for qPCR standard curve and (2) following DNase treatment, AAV standard and samples were diluted in a buffer containing 5% Tween20 for qPCR analysis. As a proof-of-concept test, a purified AAV2-GFP vector (Vigene, 5.6E12 genome copies [GC]/mL determined by ddPCR) was used as standard and was serially diluted to concentrations from 5.6E9 GC/mL to 5.6E5

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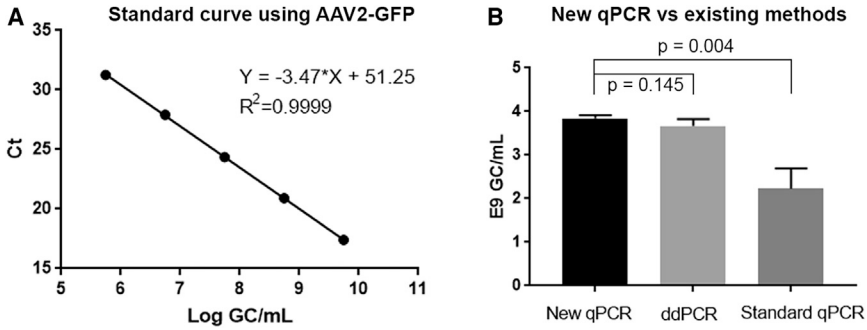


Figure 1. Testing the New qPCR Method with rAAV2
(A) After DNase treatment, an rAAV2-GFP reference standard was serially diluted in TE buffer with 5% Tween 20 from 5.6E9 to 5.6E5 GC/mL and tested by qPCR using primers and probe for GFP. A qPCR standard curve was created by plotting Ct values against the corresponding Log GC/mL. (B) The genome titer of a purified rAAV2-GFP sample was determined by qPCR referring to standard curve in (A) or by the existing ddPCR and qPCR methods. Error bar represents one standard deviation (n = 3), and statistical significance (t test) was determined between new qPCR and the other two methods.

GC/mL, followed by qPCR analysis with primer/probe targeting GFP. As shown in Figure 1A, good linearity was achieved by plotting Ct values against the corresponding GC/mL. The variations among replicates were minimal and the R^2 value was close to 1, demonstrating the feasibility of using AAV as qPCR standard. Based on three tests, the genome titer of an AAV2-GFP sample was determined to be 3.85 E9 GC/mL, which is similar to the official titer pre-determined by ddPCR (3.76 E9 GC/mL). To compare our method to existing methods, we tested the same AAV sample by ddPCR and standard qPCR. As shown in Figure 1B, new qPCR results were similar to ddPCR ($p = 0.145$) but not standard qPCR ($p < 0.05$). Accuracy of new qPCR and ddPCR methods were 102.3% and 97.6% respectively, with good inter-assay precision (RSD < 5%), which were significantly better than the 59.2% accuracy and 21.0% RSD of standard qPCR (Table 1).

To investigate whether the new qPCR method works well for other AAV serotypes and other genes of interest, we tested an AAV9 drug substance using primer/probe for the therapeutic gene. As shown in Figure 2A, the standard curve spans 5 logs from 1E10 GC/mL to 1E5 GC/mL, with minimal variations among replicates and an R^2 value equal to 1. Two AAV9 samples produced by different processes but carrying the same transgene were tested by new qPCR, ddPCR, and standard qPCR methods. As shown in Figure 2B and Table 1, new qPCR continued to perform at ddPCR-level of accuracy and precision. In contrast, although the results of standard qPCR were not significantly different, the assay precision (RSD > 20%) was much worse than new qPCR and ddPCR (RSD < 6%).

Further Characterization of the New qPCR Method

To further evaluate the new qPCR method, we investigated the inter-lab precision and robustness. Repeatability among different labs is a challenge for existing qPCR methods and large variations were reported.[8,15] We tested our method in three labs with variables including different analysts, reagents, and instruments. As shown in Figure 3A, genome titers of AAV2-GFP and AAV9-GeneX determined by 3 labs were similar and the RSDs were less than 10%. Importantly, the high precision is not due to a lack of inter-lab variation, as the starting Ct values of the standard curves could differ by as much as 0.8, or 1.7-fold by concentration. However, the impact was minimal because Ct values of AAV samples changed by almost the same degree (Figure 3B). In contrast, we failed to observe a correlation with standard qPCR (Figure S1), which results in poor assay precision. These results highlight the advantage of using AAV as qPCR standards, which resemble test samples and make the method more tolerant of experimental variables.

To assess assay robustness, AAV2-GFP was tested with half the amount of primers and probe, which mimics expiring reagents with reduced activity; or more extremely, qPCR plate was left at room temperature for 5 h before thermal cycling. As shown in Figure 4A, the genome titers measured were within an acceptable 70%−130% range compared to control, and variations among replicates stayed at a very low level (Figure 4A). Assay robustness was further assessed by testing crude cell lysate, which is known to interfere with PCR reaction.[5] A crude AAV2-GFP sample was serially diluted in TE buffer with 5% Tween 20 and tested by qPCR without proteinase treatment. While a modest matrix interference occurred at 100-fold dilution, good linearity was achieved from 1E3- to 1E5-fold dilutions (Figure 4B). These results suggest that matrix interference can be alleviated through simple dilution. To strengthen this point, we tested spike recovery in 6 different samples including crude lysate and process intermediates. As shown in Table 2, good spike recovery was achieved in all samples after 1E3 fold dilution, and the overall recovery is 102%, suggesting no matrix interference. Therefore, we conclude that the new qPCR method can be used to test unpurified AAV samples.

Testing across Different AAV Serotypes

While the method design highlights comparability between AAV standard and samples, it was not clear if they must be of the same serotype. This question is especially important for early stage gene therapy programs, which may explore several AAV serotypes, but it is cumbersome to prepare and maintain multiple standards. To this end, we purchased 6 additional AAV-GFP vectors from Vigene and determined their genome titers by ddPCR. Together with AAV2-GFP, they were diluted in assay buffer and tested as qPCR standards in the range between 1E10 GC/mL and 1E6 GC/mL. As shown in Figure 5A, there is a strong overlap among different AAV serotypes. Good linearity and little variation between replicates were observed in all 7 individual curves (Figure S2). In the same plate, three AAV-GFP samples (AAV1, 2, and 9) were tested. Remarkably, regardless

Table 1. Accuracy and Precision of New and Existing Methods

	AAV2-GFP			AAV9-Gene X (SPL1)			AAV9-Gene X (SPL2)		
Tests	New qPCR	ddPCR	SD qPCR	New qPCR	ddPCR	SD qPCR	New qPCR	ddPCR	SD qPCR
1	3.79E+09	3.58E+09	1.73E+09	3.42E+13	3.44E+13	3.55E+13	1.98E+13	2.25E+13	2.77E+13
2	3.92E+09	3.58E+09	2.29E+09	3.39E+13	3.25E+13	2.31E+13	2.02E+13	2.16E+13	2.24E+13
3	3.83E+09	3.85E+09	2.66E+09	3.54E+13	3.07E+13	2.59E+13	2.20E+13	2.22E+13	1.58E+13
Avg GC/mL	3.85E+09	3.67E+09	2.23E+09	3.45E+13	3.25E+13	2.82E+13	2.07E+13	2.21E+13	2.20E+13
% Accuracy	102.3	97.6	59.2	101.8	96.0	83.1	92.3	98.7	98.1
% RSD	1.7	4.2	21.0	2.3	5.7	23.1	5.7	2.1	27.1

One AAV2 and two AAV9 samples were tested 3 times each using new qPCR, ddPCR, and standard qPCR methods. Assay accuracy was determined by the ratio between GC/mL measured and the official titers pre-determined: 3.76E9 GC/mL for AAV2-GFP, 3.39E13 GC/mL, and 2.24E13 GC/mL for the two AAV9 samples, respectively. Assay precision was determined by the RSD of 3 tests.

of which standard curve was used, the test results were comparable and none of the samples showed preference for a standard of the same serotype (Figure 5B). Therefore, we conclude that our method is not affected by differences in AAV capsids and the same AAV standard can be used for various AAV serotypes as long as they contain the same gene of interest.

DISCUSSION

In this study, we developed a novel qPCR approach for AAV genome titer. Different from the standard qPCR methods, our approach uses an AAV material instead of plasmid DNA for standard curve. Following removal of free DNA by DNase, AAV standard and samples are simply diluted in an assay buffer containing 5% Tween 20 without the need of proteinase digestion or DNA extraction. The method has been thoroughly tested with several AAV serotypes, purified or in crude cell lysates, and was proven to be as accurate and precise as ddPCR. In contrast, standard qPCR method is significantly more variable. Importantly, the method can be automated to run in 384-well plate format, a significant advantage over ddPCR for high throughput applications.

Many analytical assays rely on a standard curve to translate raw signals into reportable values, based on the assumption that test samples resemble the reference standard within the analytical range.[16] Plasmid DNA is widely used as a qPCR standard because of the convenience to produce and characterize. However, it significantly differs from AAV in a number of ways: First, plasmid DNA is double-stranded while AAV genome is single-stranded. Second, AAV genome is encapsidated by an icosahedral shell and is less accessible than plasmid DNA. Third, AAV genome contain higher order structures (inverted terminal repeats or ITR) on both ends, which may affect primer annealing.[1] A lot of efforts in the field have been spent on improving sample preparation procedures, including proteinase digestion, viral genome extraction, restriction digestion of ITR coupled with informed primer/probe design, etc.[5–7,17] In our view, these treatments all focus on making AAV samples more comparable to plasmid DNA, yet the success of which is impossible to judge and may vary significantly among experiments. Moreover, the multi-step treatment is cumbersome and may contribute to additional assay variability. Since every AAV gene therapy program requires at least one reference material for analytical development, it is a logical choice to use that material as qPCR standard. An interesting attempt from Dobnik and colleagues was to use denatured AAV as qPCR standard, however, it did not solve the high variability problem.[2] Our method uses live AAV as standard, which is fully comparable to samples. The consumption rate is quite modest for this assay since standard curves start from a low concentration (1E10 or 1E9 GC/mL), an important consideration for gene therapy programs that are so often limited by material availability. Another advantage of using AAV is that it is known to be stable

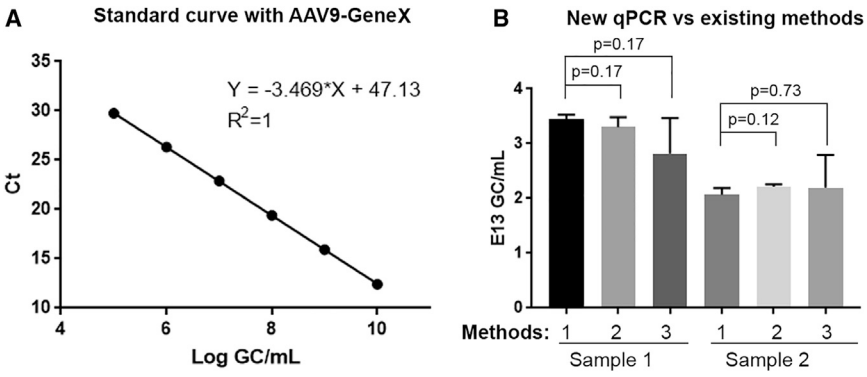


Figure 2. Testing the New qPCR Method with rAAV9

(A) After DNase treatment, an rAAV9 reference standard for a therapeutic program was serially diluted in TE buffer with 5% Tween 20 from 1E10 to 1E5 GC/mL and tested by qPCR using primers and probe targeting the transgene. A qPCR standard curve was created by plotting Ct values against the corresponding Log GC/mL. (B) The genome titers of two rAAV9 drug substances were determined by new qPCR (method 1), ddPCR (method 2), or standard qPCR (method 3). Error bar represents one standard deviation (n = 3), and statistical significance (t test) was determined between new qPCR and the other two methods.

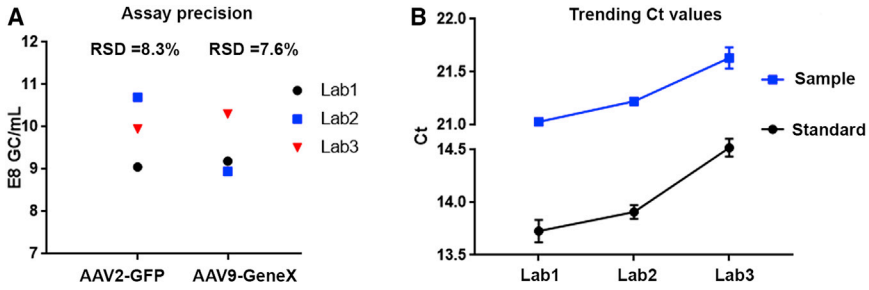


Figure 3. Inter-Laboratory Precision of AAV Genome Titers

(A) AAV2-GFP and AAV9-GeneX samples were tested by 3 analysts in 3 different labs using the new qPCR method. Relative standard deviations among different labs were calculated and were below 10% for both samples. (B) The Ct values of AAV2-GFP sample and standard curve (top dilution point) were plotted. Inter-laboratory differences existed and were consistent between sample and standard.

during long-term storage, while purified DNA is susceptible to degradation and adsorption.[12,13,18]

This work expanded the application of the "digestion-free method" we previously developed for residual DNA detection in AAV.[14] A simple dilution of AAV in TE buffer with 5% Tween 20 facilitates qPCR detection of encapsidated DNA and proteinase digestion of AAV capsid could be skipped. The addition of Tween 20 is important as other methods skipping proteinase treatment showed poor precision.[8] Our method can overcome differences in AAV capsids, as qPCR results are highly similar among 7 AAV serotypes (Figure 5). Based on this finding, only one AAV reference standard is necessary for a program, which can be used for cross-serotype testing if needed. Moreover, our method can overcome matrix interference by simply diluting out the impurities. A dilution factor of 1,000-fold or higher is possible considering the wide dynamic range of qPCR and the abundance of AAV in typical test samples. Therefore, the method can be used on both purified and crude AAV samples after appropriate dilution. In this study, primers and probe were designed for the gene of interest, which is preferred because titers of the therapeutic genes are the most clinically relevant. However, we believe it is feasible to target other parts of the viral genome that are sufficiently distant from the ITR regions. Finally, a limitation of the method is that it cannot be used to titer a new AAV material for the first time, which should be conducted by ddPCR or by existing qPCR method with multiple runs to improve accuracy. Instead, our method could faithfully propagate the initial titer to future samples and ensures batch-to-batch consistency. In conclusion, an improved qPCR method was developed that could serve as a valuable alternative to ddPCR, saving time and resource while maintaining a high assay performance level.

MATERIALS AND METHODS

rAAV Reference Material

Purified AAV-GFP vectors of 7 different serotypes were purchased from Vigene Biosciences. AAV1, 2, and 9 samples were produced by Biogen using the triple transfection platform and processed by filtration, as well as ion-exchange and affinity columns.

Primers and Probes

Sequences of PCR primers and probes for enhanced green fluorescent protein (EGFP) were ordered from Thermo Fisher Scientific Forward Primer: 5′-GTCCGCCCTGAGCAAAGA-3′; Reverse Primer: 5′-TCCAGCAGGACCATGTGATC-3′; Probe: 5′-6FAM-CCCAACGAGAAGCG-MGB/NFQ-3′.

DNase Treatment

AAV standards and samples were treated by a published method[19] with the following modifications: AAV was treated by DNase I (Thermo Fisher) at 10 units/mL in the presence of 0.05% Pluronic F68 at 37°C for 30 min.

AAV Genome Titer by ddPCR

The protocol was previously described.[19] ddPCR reactions were prepared with "ddPCR Supermix for Probes (No dUTP)" following the instructions from Bio-Rad. DNase-treated AAV samples were diluted

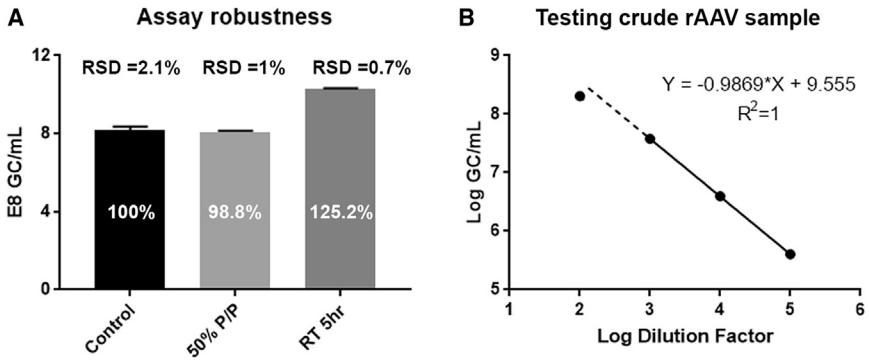


Figure 4. Assessing Assay Robustness

(A) Genome titers of an rAAV2-GFP sample was tested under regular and two stressed conditions: using half the amount of primers/probe or left at room temperature for 5 h before thermal cycling. Data were normalized by setting the result from regular condition as 100%. Error bars represent one standard deviation. (B) Overcoming matrix interference. An rAAV2-GFP crude lysate was serially diluted from 1E2 to 1E5 fold in assay buffer. The top dilution point fell out of linear range due to matrix interference, which was resolved through additional sample dilutions.

Table 2. Characterization of the New qPCR Method by Spike Recovery

Samples	Unspiked Sample (GC/mL)	Spiked Sample (GC/mL)	Spike Measured (GC/mL)	% Spike Recovery
1	6.70E+06	5.58E+07	4.91E+07	98.1
2	4.86E+06	5.56E+07	5.08E+07	101.5
3	4.93E+06	5.69E+07	5.20E+07	104
4	2.73E+06	5.45E+07	5.18E+07	103.6
5	3.34E+06	5.30E+07	4.96E+07	99.3
6	no detection	5.27E+07	5.27E+07	105.4
Accuracy				102.0%

Five rAAV2-GFP samples including 3 process intermediates (SPL 1-3), 2 crude lysate (SPL 4-5), and formulation buffer alone (SPL 6) were diluted 1E3 fold in TE buffer with 5% Tween 20, and an rAAV2-GFP reference standard was spiked into diluted samples to 5E7 GC/mL. Samples with or without AAV spike were analyzed by qPCR using rAAV2-GFP reference for standard curve. Spike measured was the difference between spiked and unspiked samples.

to an estimated titer range between 2E7 and 2E5 GC/mL. Final concentrations of primers and probe were 0.9 μM and 0.25 μM, respectively. After droplet generation, the following PCR program was run: 1 cycle of 95°C × 10'; 40 cycles of 94°C × 30", 60°C × 1', and 1 cycle of: 98°C × 10'; 4°C hold. PCR results were analyzed by QX200 Droplet reader and QuantaSoft (Bio-Rad).

Standard qPCR Method

Linearized DNA plasmid was used as qPCR standard, and DNA concentration was determined by Picogreen dsDNA kit (Molecular Probes). Plasmid was diluted in assay buffer (10 mM Tris-HCL, 50 mM KCL, 5 mM $MgCl_2$, pH = 8.0) to the range between 1E10 and 1E5 copies/mL for qPCR standard curve. AAV samples were diluted in assay buffer and sequentially treated by 10 units/μL of DNase (37°C × 30', 95°C × 10', 4°C hold) and 1 mg/mL Proteinase K (50°C × 60', 95°C × 10', 4°C hold). qPCR reactions were prepared with TaqMan Universal MasterMix in a 96-well optical plate (Life Technologies) with 1 μM primers and 0.25 μM probe. qPCR program: 1 cycle of: 50°C × 2′, 95°C × 15', 40 cycles of: 95°C × 30", 60°C × 1' was conducted in ViiA 7 Real-Time System (Life Technologies).

rAAV Genome Titer by the New qPCR Method

The dilution buffer was prepared by adding Tween 20 to Tris-EDTA (TE) buffer to a final concentration of 5%. Based on genome titers determined by ddPCR, DNase-treated AAV standards were diluted to 1E10 GC/mL followed by 5 additional 10× serial dilution to 1E5 GC/mL. DNase-treated AAV samples were diluted 10-fold or more for qPCR analysis. For spike recovery study, purified AAV standard was first diluted in TE buffer with 5% Tween 20, and then added to pre-diluted samples to a final concentration of 5E7 GC/mL.

qPCR reactions were prepared with TaqMan Universal MasterMix in a 96-well optical plate (Life Technologies). Pre-diluted rAAV standards and samples were mixed 1: 1 with qPCR reaction mixture and the final concentrations of primers and probe are 1 μM and 0.25 μM. qPCR program: 1 cycle of: 50°C × 2′, 95°C × 15', 40 cycles of: 95°C × 30", 60°C × 1' was conducted in ViiA 7 Real-Time System (Life Technologies). qPCR data were automatically analyzed by the ViiA 7 software. After defining the titer of each dilution point of the standard curve, the titers of test samples in GC/mL will be reported.

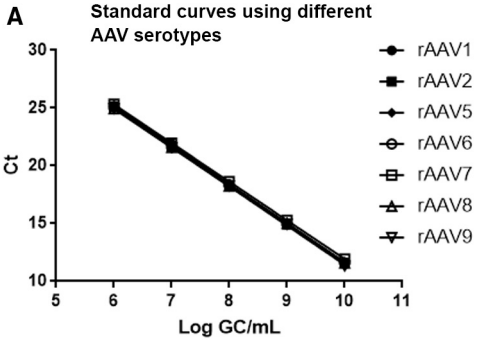
For robustness assessment, qPCR reactions were either prepared with 0.5 μM primers and 0.125 μM probe or left at room temperature for 5 h before ViiA 7 analysis. qPCR condition is the same as described above.

For inter-laboratory study, different aliquots of AAV standards and samples were sent to be tested in 3 different labs. Experiments were performed by 3 analysts on different days following the method described above.

Statistical Analysis

RSD was calculated by the following formulas:

$$\text{RSD \%} = 100\% \times \text{standard deviation(SD)}/\text{average}$$



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Standards	Samples (GC/mL measured)		
	AAV1	AAV2	AAV9
AAV1	*6.79E+08	2.72E+09	7.00E+09
AAV2	6.50E+08	*2.59E+09	6.66E+09
AAV5	7.13E+08	2.82E+09	7.23E+09
AAV6	6.92E+08	2.74E+09	7.03E+09
AAV7	7.67E+08	3.10E+09	8.07E+09
AAV8	5.82E+08	2.37E+09	6.20E+09
AAV9	6.19E+08	2.46E+09	*6.31E+09
Avg GC/mL	6.72E+08	2.69E+09	6.93E+09
% RSD	9.17	9.09	9.11

Figure 5. Comparing Different AAV Serotypes as qPCR Standard

(A) Seven different serotypes of rAAV-GFP were tested as qPCR standards from 1E10 to 1E6 GC/mL. The standard curves overlay nicely with each other. (B) Genome titers of AAV1, AAV2, and AAV9-GFP samples determined based on different standard curves. An asterisk (*) indicates a serotype match between standard and sample.

Curve fitting, t test, and regression analysis were conducted by GraphPad Prism 7.02.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.09.017.

AUTHOR CONTRIBUTIONS

Y.W. designed the experiments and wrote the paper. Y.W., N.M., and S.S. performed the experiments. M.F. and S.B. oversaw the work. All authors contributed to data analysis and interpretation.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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