A Scalable and Accurate Method for Quantifying Vector Genomes of Recombinant Adeno-Associated Viruses in Crude Lysate

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Increasing interest and application of recombinant adeno-associated viruses (rAAVs) in basic and clinical research have urged efforts to improve rAAV production quality and yield. Standard vector production workflows call for genome titration of purified vectors at the endpoint of production to assess yield. Unfortunately, quality control measures for preparations during mid-production steps and economical means to assess the fidelity of multiple batches of rAAV preparations are lacking. Here we describe a scalable and accurate method for the direct quantitative polymerase chain reaction (qPCR) titration of rAAV genomes in crude lysate. Lysate samples are pretreated with DNase I to remove vector and packaging plasmid DNAs, followed by proteinase K to release endonuclease-resistant packaged viral genomes and to proteolyze factors inherent to crude lysates that can impinge upon quantitative PCR efficiencies. We show that this method is precise, scalable, and applicable for vector genome titrations of both single-stranded and self-complementary AAV genomes irrespective of serotype differences—a major limitation for standard lysate transduction methods that indirectly screen for vector packaging efficiency. Our described method therefore represents a significant improvement to rAAV vector production in terms of alleviating time and cost burdens, in-process quality control assessment, batch/lot monitoring in large-scale preparations, and good manufacturing practices.

Keywords: crude lysate, qPCR, recombinant adeno-associated virus

INTRODUCTION

RECOMBINANT ADENO-ASSOCIATED VIRUSES (rAAVs) have gained increased appreciation as the favored delivery vehicle for therapeutic gene products for many diseases, including Canavan disease and Duchenne muscular dystrophy.^{1, 2} Many rAAV-based clinical trials for different diseases are currently being carried out worldwide.³ The treatment of lipoprotein lipase deficiency via alipogene tiparvovec (AAV1-LPL^{S447X}) is currently the only rAAV-based commercial gene therapy drug approved for human use in the world.⁴

Scalable manufacturing of rAAV and downstream processing methods to meet high vector production demands is becoming a critical bottleneck in clinical and commercial translation of rAAV gene therapeutics.^{5, 6} Many strategies aimed

to increase rAAV yield have been developed, including continuous collection of rAAV; modulating culturing temperatures after transfection;^{7, 8} and infection-based methods that employ baculovirus, herpes simplex virus, or adenovirus.⁹ Although these approaches have improved vector yield for large-scale productions, methods to accurately gauge vector yield have relied on quantitative polymerase chain reaction (qPCR) to quantify the endpoint titers of purified rAAV. Unfortunately, process development efforts devoted to scalable and reliable quantification methods during mid-manufacturing (in-process) phases of rAAV production have been stagnant.

Furthermore, the production of multiple small-scale rAAV batches (e.g., novel serotypes/variants screening) may become cost prohibitive if vector

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purification steps were required for hundreds of candidate capsids. The discovery and development of a large family of primate AAV vectors in the early 2000s, and their successful translation into clinical usage, have stimulated unprecedented efforts towards novel vector development via AAV capsid engineering. A critically important criterion for selecting ideal candidate AAV capsids is vector-packaging efficacy. This characterization is commonly performed indirectly by profiling the transduction efficiencies of packaged vectors via reporter gene expression (*e.g.*, green fluorescent protein, cellular or secreted luciferases) in tissues or cell lines. However, the transduction profiles of rAAV vectors are highly dependent on the host tissue or cell line being tested. Furthermore, many rAAVs proven to have strong *in vivo* gene transfer profiles, have weak *in vitro* transduction profiles.¹ These features therefore obscure the reliability of *in vitro* transduction assays that indirectly quantify packaging efficiencies. Alternatively, titration of packaged vector genomes in crude cellular lysate would circumvent the need to assess *in vitro* transduction as a metric for vector integrity and would directly assess packaging efficacies of assembled rAAV capsids. Additionally, the high cost of downstream processing steps for multiscale rAAV production can be significantly reduced if low-yield batches were identified with earlier monitoring steps. Quantification of rAAV vector genomes in crude lysate following vector production would therefore be invaluable for both small- and large-scale rAAV production platforms.

We have made significant optimizations to crude lysate pretreatment conditions to remove factors inherent to cellular lysates that may hamper qPCR efficiency. These factors may include proteins from the host cell as well as carryover nucleic acids originating from packaging vectors, helper plasmids, and cellular genomes. Since vector plasmids may competitively bind TaqMan probes and primers, they should be completely eliminated to obtain accurate rAAV titers. To remove any negative components of the cellular milieu from packaging cell lines, crude lysate fractions of vector preparations, were treated with DNase I, followed by proteinase K. We found that this pretreatment procedure resulted in accurate and reproducible vector genome copy quantitation in crude lysates. Overall, qPCR quantification of rAAV genome copies in crude lysate of in-process preparations can potentially save costs for large-scale rAAV production, and allow for the multiple titrations of a large number of small-scale rAAV batches without the need to perform the full vector production workflow.

MATERIALS AND METHODS

Small-scale vector production

Vectors used in this study were generated, purified, and end-point titrated using standard vector production methods as described previously.¹⁰ Briefly, small-scale production of rAAVs of different serotypes was carried out by cotransfection of 293 cells in 24-well plates with: (1) cis plasmids containing an enhanced green fluorescent protein (EGFP) transgene driven by the cytomegalovirus enhancer/chicken β -actin promoter (CB); (2) packaging plasmids with different capsid serotypes; and (3) adenovirus helper plasmids. EGFP fluorescence was observed 24 hours (h) post-transfection to assess transfection efficiency. Crude cell lysates were harvested 72 h post-transfection followed by three successive freeze–thaw cycles. The lysates of cells transfected without packaging and adeno helper plasmids were used as the negative controls for serial dilution of vector plasmid DNA to generate standard curves. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagle Medium (11965-118, ThermoFisher, Grand Island, NY) medium supplemented with 10% fetal bovine serum (10437-028, ThermoFisher) at 37°C in a humidified atmosphere of 5% CO₂.

Large-scale vector production

Large-scale rAAV preparations for self-complementary AAV7 (scAAV7) and single-stranded AAV2 (ssAAV2) vectors were purified by CsCl ultracentrifugation following standardized procedures described previously.¹⁰ Each vector preparation was generated by triple transfection of 2×10^9 HEK 293 cells.

Crude lysate pretreatment

Firstly, 5 μ L of 1 mL crude lysates containing rAAV particles were used to incubate with 50 units (U) of DNase I (Roche, Applied Sciences, Indianapolis, IN, USA) for 15 hr at 37°C, and then inactivated at 75°C for 30 min. Samples were then treated with 10 μ L of proteinase K (>600 mAU/mL) at 56°C for 2 hr, followed by incubation at 95°C for 30 min to inactivate the enzyme. The digested samples were diluted 20-fold in nuclease-free water.

Quantitative PCR

Vector plasmids were serially diluted in either nuclease-free water or negative control crude lysate from 5×10^7 to 5 copies/ μ L in 10-fold increments to generate standard curves for qPCR quantification. All qPCR reactions were composed of sample or plasmid standard, 9 μ M forward primer, 9 μ M reverse primer, and 2 μ M of probe in

TaqMan Gene Expression Mastermix (4488593, ThermoFisher) in a total reaction volume of 20 μ L each. Analyses were performed using the ViiA7 Real-Time PCR System (Applied Biosystems), and cycling conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The number of genome copies per cell was calculated using the formula: $X = [(A/B) \times 20 \times 1000] / (3 \times 10^5)$, where X is genome copies per cell, A is total quantity in the PCR reaction, B is the volume of the rAAV samples in PCR reaction, 20 and 1000 are dilution factors, and 3×10^5 is the total cell number when harvesting the crude lysate. Titers of self-complementary genomes were divided by two to allow for direct comparisons to titers of single-stranded rAAV genomes. Experiments were performed in triplicate.

Crude lysate transduction assays

Crude lysate (250 μ L) from transfections of ssAAV and scAAV preparations were used to transduce 1×10^5 cells in 24-well plates (07-200-740, Costar, ThermoFisher) in triplicate. Cells were kept in complete culture medium at 37°C and 5% CO₂ for 48 h after transduction. Blank (untransduced) and –Ad negative controls were included. Live-cell EGFP fluorescence was captured using an inverted microscope (DMI6000 B, Leica Microsystems, Buffalo Grove, IL). Images were acquired with a charge-coupled device camera (DFC365 FX, Leica) and Leica Application Suite Advanced Fluorescence software (LAS AF, v4.0.0.11706). Cells were then trypsinized and resuspended in 2% fetal bovine serum in PBS. EGFP expression was then assessed by flow cytometry using the Guave easyCyte HT system and the InCyte analysis software (v3.1) (EMD Millipore, Billerica, MA). Experiments were performed in triplicate.

RESULTS

Inhibitory effects of crude lysate assay sensitivity

To assess the efficacy and accuracy of quantitating titers during in-process stages of vector production, we first tested the inhibitory effect of crude cell lysate on target amplification during qPCR. To this end, 2×10^6 genome copies of enhanced green fluorescent protein (EGFP) plasmid was diluted in untreated negative control cell lysate or nuclease-free water and quantified by qPCR using a standard curve made in water diluent. As shown in Fig. 1A, the copies of EGFP plasmid detected in crude cell lysate (1.18×10^5) were 18.89 times lower than that of plasmid diluted in water (2.23×10^6). This result indicated that crude lysate

significantly inhibits target amplification. We hypothesized that proteins in lysates, endogenous nucleic acids, or residual plasmid vectors carried over from packaging steps may negatively impact quantification accuracy. Namely, contaminating plasmid DNA may competitively bind TaqMan probes/primers. To directly address these concerns, crude lysates were treated with either DNase I, proteinase K, or both. EGFP copies detected in each treatment group were 1.12×10^6 (proteinase K), 359.44 (DNase I), and 191.19 (DNase I plus proteinase K) (Fig. 1B). These findings indicate that DNase I treatment can nearly deplete all plasmid DNA in crude lysates, and proteinase K digestion can eliminate any inherent inhibitory proteins.

We next assessed the impact of crude lysate treatment with extended DNase I and proteinase K digestions on qPCR accuracy. Vector plasmid DNA was serially diluted in nuclease-free water, or in lysates from nontransfected cells (negative crude lysate) treated with DNase I/proteinase K digestion, to compare the standard curves of target between the two dilution conditions. As illustrated in Fig. 1C, there is little to no observable difference between quantification of EGFP copies diluted in crude lysate and water. The resulting qPCR metrics (Table 1) were obtained from samples assayed on the same plate in triplicate. The amplification efficiencies for the standard curves in water and in crude lysates were 102.21% and 98.64% respectively. Moreover, no significant difference was observed between the slopes of the two standard curves using linear regression analysis ($F = 0.012$, $DF_n = 1$, $DF_d = 11$, $p = 0.91$). Importantly, detection of 100 copies of plasmid was within the linear range of target detection with both water and crude lysate diluents (Fig. 1C). These findings suggest that qPCR can detect DNA templates in crude lysate with relatively high accuracy and sensitivity.

Titration of treated crude lysate by qPCR is applicable for different vector-serotype preparations and is reproducible across independently packaged vectors

We next wished to demonstrate the sensitivity and feasibility of qPCR titer detection in treated crude lysates for multiple small-scale preparations, as would be the case for screening the packaging efficacies for newly discovered or designed capsids sequences. To simulate the process of testing multiple candidate rAAV vectors based on novel capsid designs, ssAAV and scAAV vectors containing the EGFP reporter transgene were packaged into different rAAV serotypes. Altogether, rAAV1, 2, 3b, 4, 5, 6, 6.2, 7, 8, 9, rh.8, rh.10, and rh.43 vectors were

Figure 1. Crude lysate inhibits qPCR detection of target DNA. **(A)** EGFP plasmid DNA (2×10^6 copies) was diluted in water or undigested cell lysate ($200 \times$ dilution) and quantitated by qPCR. **(B)** Proteinase K treatment significantly reduces the inhibitory effect of cell lysate on PCR amplification. Crude lysates were treated with DNase I (37°C for 15 h), proteinase K (56°C for 2 h), or both. **(C)** Comparisons of qPCR standard curves made by diluents of water and DNase I/proteinase K-digested negative crude. The log of standard DNA dilutions in copy number was plotted against Ct values. EGFP, enhanced green fluorescent protein; Ct, cycle threshold; qPCR, quantitative polymerase chain reaction.

produced as previously described.¹⁰ Expression of the EGFP reporter by fluorescence suggested comparable transfection efficiencies among all groups (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb). As shown in Fig. 2A, differences in rAAV titers between the serotypes tested were within or near 10-fold. Notably, all ssAAV vectors showed ~ 10 -fold greater titers than scAAV vectors. The simultaneous quantification of multiple serotypes, packaging both ssAAV and scAAV genomes, demonstrates that

qPCR quantification of rAAV in crude lysate can be performed on multiple samples in parallel.

To further assess the accuracy and reproducibility of using crude lysate treatments to achieve reliable qPCR titers, 10 individual, small-scale scAAV2/2 viral preparations were produced and assayed. As illustrated in Fig. 2B, no significant differences in rAAV titers were observed among the 10 replicate crude lysate samples treated with sequential DNase I and proteinase K digestions. These data suggest that the near complete removal of inhibitory factors

Table 1. Characteristics of qPCR standard curves that were made by serially diluting enhanced green fluorescent protein plasmids in water or in DNase I/proteinase K digested crude lysate

	Standard Made in Crude Lysate Diluent	Standard Made in Water Diluent
<i>Best-fit values</i>		
Slope	-3.269 ± 0.05859	-3.191 ± 0.04835
Y-intercept when $X=0.0$	38.05 ± 0.3155	37.34 ± 0.2604
X-intercept when $Y=0.0$	11.64	11.7
1/slope	-0.3059	-0.3134
<i>95% confidence intervals</i>		
Slope	-3.420 to -3.119	-3.315 to -3.066
<i>Goodness of fit</i>		
R square	0.9984	0.9989
Sy.x	0.3101	0.2558
Equation	$Y = -3.269X + 38.05$	$Y = -3.191X + 37.34$
Amplification factor	1.99	2.02
qPCR Efficiency	98.64%	102.21%

inherent to cellular lysate can yield highly reproducible qPCR titers of rAAV genomes.

Assessment of packaging efficiency by crude lysate qPCR is more reliable than indirect quantification by *in vitro* transduction assays

The ability to determine titers from treated crude lysates allows investigators to accurately

quantitate packaging efficiencies during in-process steps of vector production, especially during the process of screening libraries of novel AAV capsids for rAAV production. This ability circumvents the need to rely on approaches that indirectly assess packaging efficiency by *in vitro* transduction assays. These methods involve transduction of cell lines with crude lysate and the quantification of fluorescent reporter genes. Unfortunately, lysate transduction is unreliable for rAAV serotypes that have incompatible tropism profiles with certain cell lines. We directly demonstrated this unreliability by transducing HEK 293 cells with aliquots of untreated crude lysate used for previous qPCR titration assays (Fig. 3 and Supplementary Fig. S2). We also compared transduction efficiencies of lysates to the efficiency of infection by purified scAAV2-CB6-EGFP vectors. To obtain accurate quantitation of reporter gene expression, we chose to measure EGFP fluorescence in transduced cells by flow cytometry (Supplementary Fig. S3). In summary, although transfection of increasing dosages of purified rAAV in HEK 293 cells led to a very linear correlation in EGFP fluorescence (Fig. 3A), expression by cells transduced with crude lysate

Figure 2. Quantification of rAAV titers by qPCR. **(A)** Titers of ssAAV and scAAV vectors were determined by comparison to both standard curves of water (black bars) and DNase I/proteinase K digested crude lysate (gray bars). **(B)** The reproducibility of qPCR was tested by titrating 10 independent scAAV2/2 vector preparations from pretreated crude lysates. Values reported are GC per cell. AAV, adeno-associated viruses; scAAV, self-complementary AAV; ssAAV, single-stranded AAV; GC, genome copies.

Figure 3. Accuracy of EGFP quantification by flow cytometry assessment is limited by transduction efficiency differences between serotypes. **(A)** Bar graph of EGFP measurements of HEK 239 cells (uninfected control) and cells infected with rAAV2/2 at multiplicity of infection dosages of 1E2, 1E3, and 1E4. The y-axis displays the average green fluorescent signals (GRN-HLog) by representative transduced cell population groups indicated on the x-axis. **(B)** Detection of EGFP fluorescence of ssAAV-EGFP or scAAV-EGFP crude lysate transduced cells. Each rAAV serotype is represented by ssAAV and scAAV vector genome types. The dotted gray line indicates background fluorescence detection as defined by the -Ad negative control. **(C)** Transduction efficiencies of crude lysates calculated by the average EGFP fluorescent signal over the genome copies detected per cell (GC/cell). rAAV, recombinant AAV.

did not correlate well with the genome copies detected by qPCR analysis (Fig. 3B, C). The differences in detected genome copies among single-strand or self-complementary groups were within 10-fold, whereas the detection of EGFP fluorescence from crude lysate transduced cells were much greater. For example, ss/scAAV4 samples were detected at near background levels, while ss/scAAV2 samples displayed ~100-fold over background. To better illustrate the discrepancy between assessments by qPCR of crude lysate versus crude lysate transduction followed by reporter gene quantitation, we normalized the expression of EGFP detected via flow cytometry by the abundance of genome copies detected per cell as assessed via qPCR across samples (Fig. 3C). These results demonstrate well the tropism differences for HEK 293 cells between different serotypes, and show the higher efficiency for transgene expression by scAAVs. Importantly, we highlight the inaccuracy of crude lysate transduction for gauging packaging efficiency due to these factors

Estimation of vector recovery from downstream processing and purification of large-scale rAAV preparations

One clear benefit of being able to determine rAAV titers following crude cellular lysate is the ability to compare in-process titers to endpoint titers to estimate vector loss during production. To demonstrate this, crude lysates from large-scale preparations of one ssAAV2 and one scAAV7 were obtained and treated with the aforementioned sequential DNase I and proteinase K digestions. rAAV titers in treated lysates were compared to titers of purified virus from the same production batches, and the viruses were purified by CsCl ultracentrifugation. As expected, the total viral titers in crude lysates were higher than those of the purified vectors (Fig. 4). This demonstration lends proof that quantification of vector genomes in crude lysate can be quite useful in assessing or predicting the productivity of a particular vector batch and provides valuable information towards improving downstream processing for vector recovery.

DISCUSSION

Quantification methods that rely on qPCR and digital droplet PCR are widely used to assess the genome titers of purified rAAVs,¹¹ yet little effort has been made to develop methods for titration of ssAAV and scAAV in crude cellular lysate. Previously reported methods for crude lysate titration

Figure 4. Estimation of vector loss in large-scale rAAV productions. The total viral titers of ssAAV and scAAV vectors recovered from crude lysates were evaluated using standard dilutions in negative crude lysate, while titers of purified viruses were determined by using standard dilutions in water.

are underutilized and, practically speaking, unreliable and not easily implemented. In this report, we describe the quantification of rAAV in crude lysates following rAAV vector production by HEK 293 triple-transfection (Fig. 5). PCR inhibition is a major concern for qPCR accuracy, and previous studies have shown that components in crude lysates can dramatically inhibit qPCR amplification.¹² To accurately quantify rAAV titers in crude lysate, these repressive factors must be thoroughly removed. Our findings suggest that inconsistencies for qPCR are related to the abundance of inhibitory protein factors that can be efficiently removed by extended proteinase K treatments. The efficiency of qPCR using crude lysate was markedly elevated

Figure 5. Flowchart of rAAV titration in crude lysates using DNase I and proteinase K pretreatment.

after 2 h of proteinase K digestion, (Fig. 1A, B). DNase I treatment is essential to ensure that any genome copies detected by qPCR are from encapsidated and DNase I-resistant vector genomes alone, and not from residual transfected plasmid DNA. Unlike qPCR assays using Master SYBR Green system, TaqMan qPCR assays used in our study further reduces the detection of nonspecific amplicons, and provides a more accurate and reproducible rAAV titration.¹³

Although there was no significant difference between the slopes of the standards diluted in crude lysate versus water (Table 1), the cycle threshold values of crude lysate were slightly higher than water (Fig. 1) at lower ranges of the standard (10^2 – 10^3). This minor difference may be due to inhibitory factors in crude lysate that cannot be completely eliminated by DNase I and proteinase K digestion. Additional optimization of crude lysate treatment may therefore further improve qPCR accuracy at very low ranges. At present, droplet digital PCR (ddPCR) has emerged as a powerful technique for the absolute quantification of purified rAAV.¹⁴ This alternative method would abolish the need to compare to a standard curve for accurate quantitation and would further improve the sensitivity of in-process titration of crude lysates following our pretreatment protocol.

To demonstrate the utility and robustness of the described method in screening a large number of capsids for vector packaging efficiencies, we simultaneously titrated 13 serotypes of ssAAV and scAAV in crude lysate and show that assessment of vector productivity across diverse serotypes/variants can be scaled to profile multiple small vector preparations in parallel. In addition, we show that the assay can reproducibly titer multiple batches to accurately estimate vector loss in the downstream processing of large-scale rAAV preparations (Figs. 2 and 4). Interestingly, we see a significantly decreased vector genome titer in purified scAAV7 that is not observed of ssAAV2. This observation may be explained by inherent differences between rAAV2 and other rAAV serotypes. As previously reported, rAAV2 tends to stay in-

tracellular, whereas other serotypes tend to release into culture medium after packaging.¹⁵ Crude lysate quantification will detect all packaged vector genomes, whereas after purification by the conventional method, other rAAV serotypes that are released into the culture medium are not efficiently recovered.

In summary, our described method fulfills unmet needs for quantifying vector genomes in crude lysates from both large- and small-scale rAAV preparations in a scalable, sensitive, and reproducible manner. Importantly, our described qPCR protocol represents a key process development for in-process quality control and batch/lot monitoring in large-scale, good manufacturing practice preparations. Since titers can be detected in advance, quantification of rAAV in crude lysate will mitigate unnecessary costs associated with the purification of low-yield batches. The ability to quantitate rAAV in crude lysate can also be beneficial for screening novel serotypes/variants for their ability to successfully encapsidate vector genomes without the need to purify virus. All of these aspects further enhance the use of rAAV in clinical applications and basic research.

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AUTHOR DISCLOSURE

Guangping Gao is a cofounder of Voyager Therapeutics and holds equity in the company and is an inventor listed on patents with potential royalties licensed to Voyager Therapeutics and other biopharmaceutical companies. The remaining authors have nothing to disclose.

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