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Original Article

Development of a one-step RT-ddPCR method to determine the expression and potency of AAV vectors

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Robust assays to quantify adeno-associated virus (AAV) vector expression and potency are essential for gene therapy development. These assays inform the efficacy, safety, and pharmacodynamic profiles of AAV development candidates. Additionally, for gene downregulation strategies such as RNAi, knockdown of endogenous genes reflects the mechanism of action of such development candidates. Therefore, a method to quantify target mRNA repression is necessary for measuring vector potency both *in vitro* and *in vivo*. Here, we report the development of a one-step reverse-transcription droplet digital PCR (RT-ddPCR) method to analyze expression of AAV vectors and the potency of AAV-RNAi vectors. This one-step RT-ddPCR method simplifies the workflow, allows for duplexing reactions, and enables absolute quantification of transcripts without standard materials. With a gene augmentation vector, we demonstrate the application of RT-ddPCR in quantifying vector expression *in vitro* and in non-human primate (NHP) samples. This novel method is demonstrated to be precise and linear within the range of 0.05–25 ng of RNA input. Using an AAV-RNAi vector, we further demonstrate the utility of this RT-ddPCR method in quantifying potency. Orthogonal potency assays, including ELISA and functional readout, correlate well with RT-ddPCR results. Therefore, one-step RT-ddPCR can be implemented in the analytical and pharmacological characterization of AAV vectors.

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is the vector of choice for gene transfer in research and central nervous system (CNS) gene therapy drug development.[1] AAV vectors contain two components: an icosahedral protein capsid that interacts with cell surface receptors and therefore determines tissue tropism and a DNA genome that encodes an engineered therapeutic transgene and regulatory elements flanked by two inverted terminal repeats (ITRs).[2] In both research applications and drug development, key vector attributes, such as titer, purity, and potency, are critical parameters for *in vivo* pharmacology studies. When characterizing AAV vectors *in vivo*, nucleic acid-based quantitative assays play a pivotal role in determining the biodistribution, transgene expression profiles, and target engagement. All these assays use AAV transgenes or transgene products to quantify the exposure of AAV vectors in animal models. Data from these assays are used in pharmacokinetics-pharmacodynamics (PKPD) modeling, vector shedding characterization, and dose projection for clinical trials.

Quantitative PCR (qPCR) and quantitative reverse transcription PCR (qRT-PCR) are widely adopted for vector titering assays, biodistribution, and transgene expression assays.[3,4] However, serial dilution of standard materials containing the target amplicon sequence is necessary to establish standard curves for each qPCR run for vector titering. Preparation of well-calibrated and -characterized standard curves is laborious and poses several challenges, including concentration value assignment and ensuring retention of large quantities for long-term use. Additionally, the nature of the qPCR method can give rise to variation due to pipetting errors and inter-lab discrepancies.[5] Furthermore, changing standards during the life cycle of an assay or a drug development program has proven to be challenging. Droplet digital PCR (ddPCR) combines microfluidics and probe-based qPCR technologies to enable absolute quantification of nucleic acids.[6] In each ddPCR reaction, water-oil emulsions separate target nucleic acid molecules into ~20,000 droplets, partitioning each reaction into small numbers of molecules per droplet. Each droplet is a separate vessel for a single PCR amplification event with a binary positive or negative readout. Poisson statistical analysis of the readouts from all the droplets determines the concentration of target template in the original test samples. Because of this digital, binary readout, no standard curve is required for ddPCR assays to enable quantification. In addition, other major

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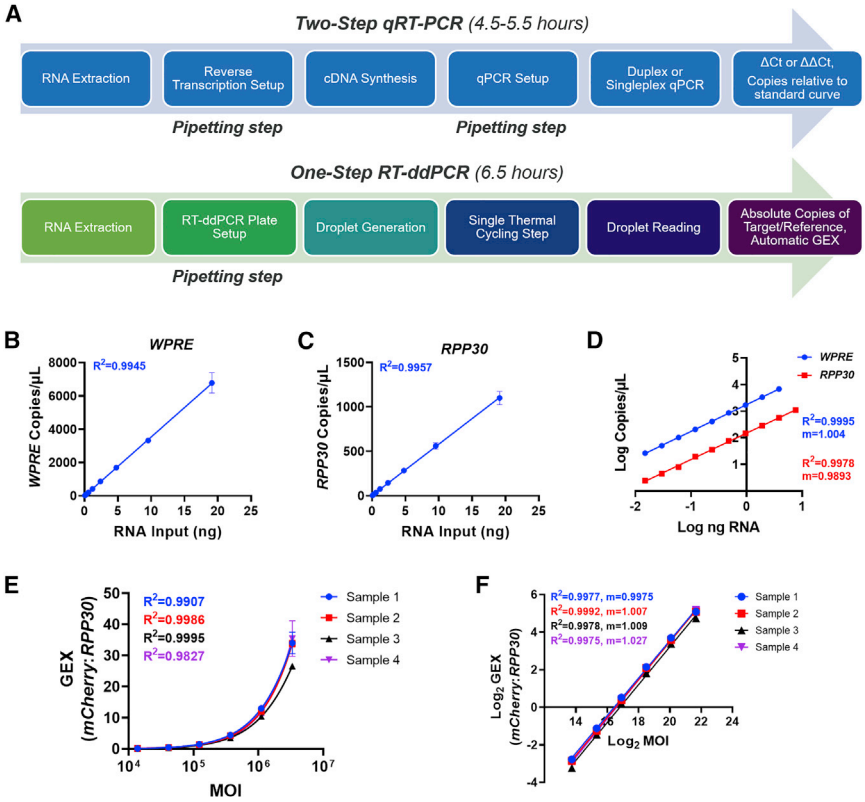

Figure 1. Quantification of relative AAV vector expression using one-step RT-ddPCR

(A) Comparison of workflows in one-step RT-ddPCR and two-step qRT-PCR. (B) Linearity of *WPRE* mRNA readouts in a duplex one-step RT-ddPCR assay coupled with an *RPP30* primer/probe set. N = 3 replicates for each RNA input. (C) Linearity of *RPP30* mRNA readouts in a duplex one-step RT-ddPCR assay coupled with a *WPRE* primer/probe set. N = 3 replicates for each RNA input. (D) Log_2-log_2 transformation of (B) and (C), indicating linearity and high sensitivity of the duplex one-step RT-ddPCR assay for *WPRE* and *RPP30*. (E) Application of one-step RT-ddPCR in analyzing AAV-CAGGs-mCherry expression in transduced HeLa cells at MOIs ranging from 1.37×10^4 vg/cell to 3.33×10^6 vg/cell. (F) Log_2-log_2 transformation of (E) to demonstrate linearity. N = 3 independent transduction replicates, each with individual RT-ddPCR result. Error bars indicate standard deviation. GEX, relative gene expression; m, slope.

advantages of ddPCR include high precision and reduced PCR bias.[7] As a result, ddPCR has been widely adopted as a method to quantify AAV titers in industry in recent years.[4,8–10]

In this study, we developed a one-step reverse transcription ddPCR (RT-ddPCR) method for AAV analytical and pharmacological assays. First, using an AAV-mCherry overexpression vector, we demonstrate that one-step RT-ddPCR can be used to quantify vector expression *in vitro* and *in vivo*, with a simplified protocol and robust duplexing capability. Furthermore, in the context of a gene repression vector, we applied one-step RT-ddPCR to quantify both vector expression and potency. In this case, orthogonal potency methods were used to show robust correlation between protein expression and protein function readouts and the target gene downregulation measured by one-step RT-ddPCR. In addition, results generated by the one-step RT-ddPCR method were compared to those generated by standard two-step RT-PCR methods.

Given its absolute quantification nature and increasing automation capabilities, ddPCR has gained popularity in cancer and infectious disease diagnostics.[11–17] Particularly in the AAV gene therapy field, titration using ddPCR has been identified as the industry standard during the last decade.[4,8,10] We believe that one-step RT-ddPCR can also be widely applied in the pharmacology studies of both gene augmentation and gene repression therapies.

RESULTS

Quantification of AAV vector expression using duplex one-step RT-ddPCR

Not all cells with the same number of AAV vector genomes express AAV transgenes at the same level. Vector expression also depends on cell type, vector endocytosis pathway, and nuclear entry.[18,19] Thus, understanding AAV vector expression profiles is a critical step in vector characterization. Compared to quantifying transgene mRNA expression levels with a two-step qRT-PCR method, one-step RT-ddPCR has a considerably more simplified bench workflow and data analysis process. It starts with AAV transduction and RNA extraction from cells or animal tissues (Figure 1A). Total RNA is then combined with one-step RT-ddPCR reaction mix and two primer/probe sets for the target and housekeeping reference genes. Droplets are produced from each reaction well on an automated droplet generator via a microfluidic system that separates mRNA molecules into individual emulsified droplets, each of which functions as a nano-scale RT and subsequent PCR reaction. After droplet generation, the plate is transferred to a thermal cycler for endpoint RT-PCR. The cycle starts with a 60-min reverse transcription step at 42°C–50°C, followed by PCR amplification. Both reverse transcription and subsequent PCR use the same primers present in the target and reference detection primer/probe sets. Data outputs are based on Poisson statistics of the number of fluorophore positive and negative droplets in each well. In our applications, detection of target gene and *RPP30*, a housekeeping gene, is duplexed in the one-step RT-ddPCR reactions to allow for appropriate internal control. In contrast, an qRT-PCR workflow could be more complex if the operator performs standard two-step qRT-PCR with separate RT and qPCR steps. There are more pipetting steps, which increases variability as well as chances of contamination in reactions. Additionally, qRT-PCR data analysis requires

Table 1. Linearity and sensitivity of *WPRE* and *RPP30* duplex one-step RT-ddPCR

RNA input (ng)	Average measured *WPRE* copies/μL	%CV	Average measured *RPP30* copies/μL	%CV	GEX (*WPRE:RPP30*)	Average GEX (*WPRE:RPP30*)	%CV
19.1	6,780	9.0	561	6.3	12.1		
9.56	3,323	3.2	284	3.6	11.7		
4.78	1,692	2.8	146	1.7	11.6		
2.39	860	4.6	76.2	2.2	11.3		
1.195	410	5.2	36.1	2.5	11.4	11.5	6.0
0.598	206	3.2	19.4	8.2	10.6		
0.299	103	3.4	8.10	13.0	12.7		
0.149	50.5	1.5	4.50	13.9	11.2		
0.075	26.5	4.5	2.53	16.0	10.5		

GEX, relative gene expression.

the operators to convert raw cycle threshold (Ct) values into ΔΔCt and then percentage of change in expression, whereas RT-ddPCR generates absolute quantifications of both target and housekeeping mRNA, as well as the relative expression of the target to the reference mRNA. This relative gene expression analysis allows for more robust comparison between studies.

To assess the linearity and determine the sensitivity of the one-step RT-ddPCR assay, HeLa cells were transduced with an AAV vector containing the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) sequence. Total RNA was extracted, and its concentration in ng/μL was determined by NanoDrop UV absorbance. The total RNA was then serially diluted and analyzed in duplex one-step RT-ddPCR with primer/probes for *WPRE* and *RPP30*. Both *WPRE* and *RPP30* demonstrate good linearity in the duplex reaction, with R^2 values of 0.9945 (Figure 1B) and 0.9957 (Figure 1C), respectively. Each target also demonstrates high sensitivity and good precision between replicates as well as dilutional linearity of relative gene expression in the duplex reaction, with a linear range of 0.075 to 19.13 ng of RNA input (Table 1; Figure 1D).

To further evaluate the application of RT-ddPCR in AAV expression analysis, we constructed an mCherry reporter vector as a tool. Specifically, a proviral vector was generated by subcloning mCherry coding sequence and WPRE into an AAV proviral plasmid with a CAGGs promoter and a human growth hormone (hGH) poly-adenylation signal. This plasmid was then packaged into AAV9 and AAVrh10 capsids to produce AAV-CAGGs-mCherry. By transducing HeLa cells with a series of multiplicities of infection (MOIs) of various batches of the AAV-CAGGs-mCherry vector, expression dose-response curves were generated from a one-step RT-ddPCR duplex reaction of *mCherry* and *RPP30*. With a three-parameter logistic (3PL) sigmoidal curve fitting, four independent samples yield curves with R^2 values of >0.98 (Figure 1E). With linear curve fitting of the $\log_2$-$\log_2$ transformation of the MOI series and relative gene expression (GEX) values, all four samples yield curves with R^2 values > 0.99 and slopes of 1.00 (Figure 1F).

Next, this one-step RT-ddPCR method was applied to quantifying vector expression of AAV9-CAGGs-mCherry and AAVrh10-CAGGs-mCherry *in vivo*. In short, adult cynomolgus monkeys were dosed with 1.5×10^{14} viral genomes (vg) of AAV9 or AAVrh10 vectors encapsulating the CAGGs-mCherry-WPRE-hGHpolyA transgene via intracisternal magna (ICM) route of administration. Twelve to fourteen days post-injection, animals were euthanized to collect tissue samples from multiple regions of the brain, including frontal cortex, motor cortex, temporal cortex, parietal cortex, occipital cortex, cerebellum, amygdala, hippocampus, thalamus, substantia nigra, caudate putamen, locus coeruleus, and medulla oblongata. Total RNA was extracted and underwent duplex one-step RT-ddPCR analysis with *mCherry* and *RPP30* primer/probes and two-step qRT-PCR analysis with a *WPRE* primer/probe. The *RPP30* primer/probe set detects a region of 100% homology between human and cynomolgus *RPP30* cDNA. The results of the one-step RT-ddPCR are reported as copies of *mCherry* mRNA relative to copies of *RPP30* mRNA (Figure 2A). To compare the one-step RT-ddPCR method to the more commonly used two-step qRT-PCR assay, the same RNA samples were first reverse-transcribed by *WPRE* target-specific primer, followed by qPCR with a *WPRE* primer/probe set. The results are reported as *WPRE* mRNA copy numbers per 10 ng of total input RNA (Figure 2B). Linear correlation between the one-step RT-ddPCR and the two-step qRT-PCR results was calculated by plotting the $\log_{10}$ transformation of each dataset. The two methods show good correlation, with an R^2 of 0.9496 and a slope of 0.9794 (Figure 2C).

Quantification of endogenous gene knockdown by an AAV repression vector using duplex one-step RT-ddPCR

To expand the application of this one-step RT-ddPCR method, we sought to utilize it to determine the potency of AAV vectors that deliver an RNAi element for gene repression. Two different proviral plasmids were cloned to contain an RNAi component controlled under a ubiquitous promoter, as well as the WPRE sequence. These plasmids were then packaged into AAV capsids to produce AAV-RNAi vectors that specifically knock down superoxide dismutase 1 (*SOD1)* gene expression. To first confirm expression of the transgene

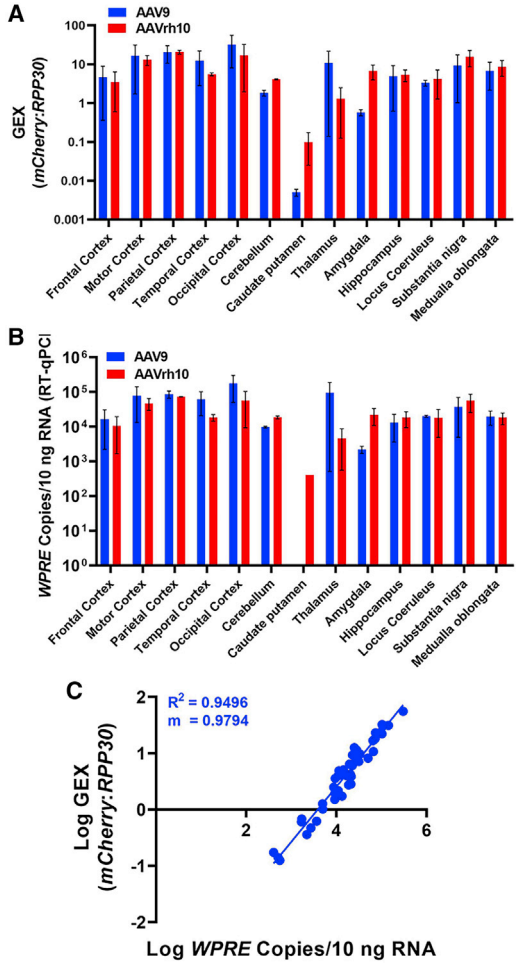

Figure 2. Expression of AAV9-CAGGs-mCherry and AAVrh10-CAGGs-mCherry vectors in NHP brains

Adult cynomolgus macaques were dosed with 1.5 × 10^{14} vg/NHP of AAV9-CAGGs-mCherry or AAVrh.10-CAGGs-mCherry vectors via ICM route of administration. Animals were euthanized on days 12–14 post-administration, and brain samples were analyzed for transgene expression. (A) Vector expression measured by duplex one-step RT-ddPCR in the format of GEX of *mCherry* mRNA copy numbers to *RPP30* mRNA copy numbers. (B) Vector expression measured by two-step qRT-PCR in the format of *WPRE* mRNA copy numbers per 10 ng of total RNA. (C) Correlation of the results generated by the one-step RT-ddPCR and two-step qRT-PCR methods. N = 2 animals per group. Each animal sample was tested with 2 technical replicates in RT-ddPCR and 3 technical replicates in qRT-PCR. Error bars indicate standard error of the mean for each group.

from the AAV-RNAi vectors, HeLa cells were transduced with an MOI series, and expression was measured using the duplex one-step RT-ddPCR with *WPRE* and *RPP30* primer/probe sets. Three different batch preparations of one AAV-RNAi vector were tested, along with two different batch preparations of the second vector. With a 3PL sigmoidal curve fitting, the five independent samples yield curves with R^2 values of >0.95 (Figure 3A). With linear curve fitting of the $\log_2$-$\log_2$ transformation of the MOI series and GEX values, all five samples yield curves with R^2 values > 0.98 (Figure 3B).

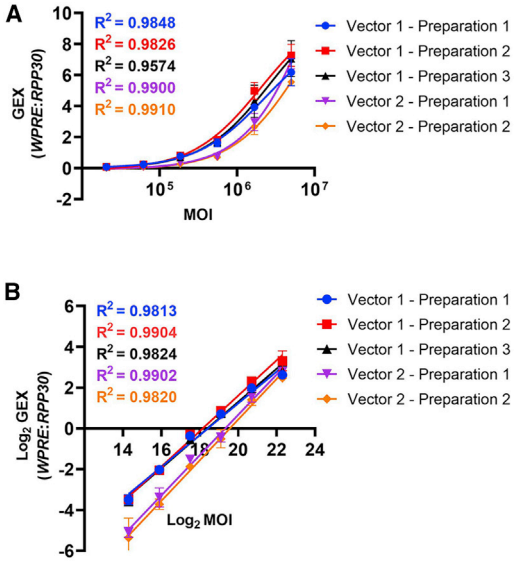

Figure 3. Expression of AAV-RNAi vectors in transduced HeLa cells

(A) Dose response of relative GEX in AAV-transduced HeLa cells with MOIs ranging from 2.06 × 10^4 vg/cell to 5.00 × 10^6 vg/cell. Five lots of two different AAV vectors were assayed. RNA was extracted ~72 h post-transduction. Gene expression of *WPRE*-containing mRNA relative to housekeeping gene *RPP30* mRNA copy numbers was quantified by a duplex one-step RT-ddPCR method. (B) Log_2-$\log_2$ transformation of (A) to demonstrate linearity. N = 3 independent transduction replicates, each with individual RT-ddPCR result. Error bars indicate standard deviation.

Since the AAV-RNAi vectors knock down the endogenous *SOD1* gene at the mRNA level, the one-step RT-ddPCR method could be utilized to measure not only the expression but also the potency of these vectors. To assess the linearity and determine the sensitivity of the one-step RT-ddPCR assay for measuring *SOD1* knockdown, total RNA was first extracted from naive HeLa cells and the RNA concentration in ng/μL was determined by NanoDrop UV absorbance. The total RNA was then serially diluted and analyzed in duplex one-step RT-ddPCR with primer/probes for *SOD1* and *RPP30*. Both *SOD1* and *RPP30* demonstrate good linearity in the duplex reaction, with R^2 values of 0.9996 (Figure 4A) and 0.9993 (Figure 4B), respectively. By plotting the $\log_{10}$ of RNA input per reaction versus the $\log_{10}$ copies/μL result, each target also demonstrates high sensitivity and good precision between replicates as well as dilutional linearity of relative gene expression in the duplex reaction, with a linear range of 0.049 to 25 ng of RNA input (Table 2; Figure 4C). Furthermore, HeLa cells were demonstrated to have *SOD1* expression ~8-fold higher relative to *RPP30* expression. This allows for the potential to measure greater than 3 logs of reduction of *SOD1* expression, without going below the detection of the ddPCR. Based on this, it was determined that HeLa cells were a suitable AAV-RNAi potency assay system for assessing the knockdown of *SOD1* expression.

To further demonstrate the assay suitability, a preliminary qualification was performed to assess the accuracy, precision, and linearity of

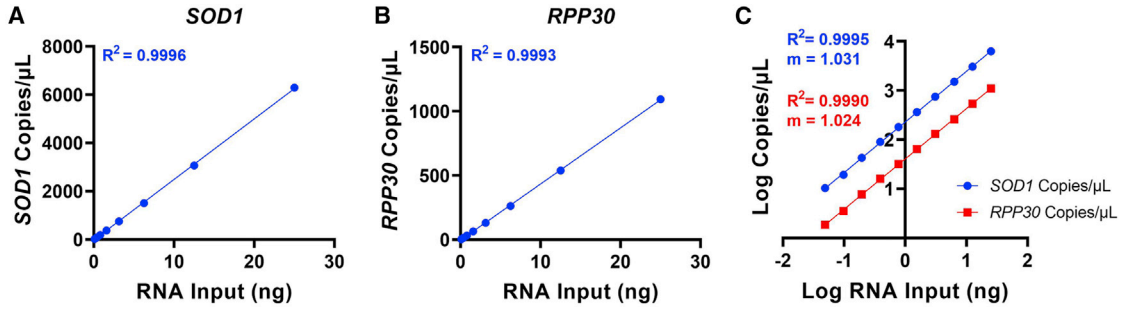

Figure 4. Sensitivity of the duplex one-step RT-ddPCR assay with *SOD1* and *RPP30* primers and probes

(A) Linearity of *SOD1* mRNA readout in a duplex one-step RT-ddPCR assay coupled with an *RPP30* primer/probe set. RNA was extracted from naive HeLa cells. (B) Linearity of *RPP30* mRNA readout in a duplex one-step RT-ddPCR assay coupled with a *SOD1* primer/probe set. (C) Log-log transformation of (A) and (B), indicating linearity and high sensitivity of the duplex one-step RT-ddPCR assay for *SOD1* and *RPP30*. N = 3 independent transduction replicates, each with individual RT-ddPCR result. Error bars indicate standard deviation. Some error bars are too small to be visible.

the method as an AAV-RNAi vector potency assay. One of the AAV-RNAi vectors was used to prepare different MOI series with different expected % relative potencies (%RPs) compared to the reference, defined as 100%. This was performed in two independent assays. All tested %RP values recover well, with an average % recovery or accuracy of 98.0% (Table 3; Figure 5A). The precision was also calculated based on the % recoveries and is determined to be 1.4%, demonstrating high precision. When the expected %RP and measured %RP values are plotted, the assay shows good linearity, with an R^2 of >0.99 and a slope of 1.15. To assess the reproducibility of the assay, the same AAV-RNAi vector was tested in 4 independent assays. By using the first run as the reference, the run-to-run variability was determined by calculating the %RP of the three additional runs (Figure 5C). All values are within the range of 80%–125%, demonstrating good reproducibility in the assay.

To confirm the utility of the one-step RT-ddPCR method as an appropriate potency assay, we compared it to a series of orthogonal methods. The first step was to compare it to the standard two-step qRT-PCR, as well as a two-step RT-ddPCR. Both methods require a separate, initial reverse transcription step prior to quantification by qPCR or ddPCR. Using the same extract of RNA from the cell-based portion of the assay, the three methods were compared for measuring %*SOD1* knockdown using duplex *SOD1* and *RPP30* reactions (Figure 6A). The one-step RT-ddPCR shows good correlation to both two-step qRT-PCR, with an R^2 value of 0.9998, and the two-step RT-ddPCR, with an R^2 value of 0.9993 (Figure 6B). The second assay type compared to the one-step RT-ddPCR method was an ELISA to measure SOD1 protein knockdown (Figure 6C), which shows good correlation, with an R^2 of 0.9523 (Figure 6D). The last assay compared to the one-step RT-ddPCR assay was a colorimetric assay to measure knockdown of SOD1 activity (Figure 6E), which also shows good correlation, with an R^2 of 0.9787 (Figure 6F).

Finally, we compared the one-step RT-ddPCR to two-step qRT-PCR as an *in vivo* potency measurement of AAV-RNAi vectors. Total RNA was extracted from SOD1-G93A mouse tissue from animals treated with the *SOD1* AAV-RNAi vectors. RNA samples were diluted in

Table 2. Linearity and sensitivity of *SOD1* and *RPP30* duplex one-step RT-ddPCR

RNA input (ng)	Average measured *SOD1* copies/µL	%CV	Average measured *RPP30* copies/µL	%CV	GEX (*SOD1:RPP30*)	Average GEX (*SOD1:RPP30*)	%CV
25.0	6,287	0.9	1,093	2.3	5.75		
12.5	3,063	2.4	539	3.1	5.69		
6.25	1,504	1.2	261	2.1	5.76		
3.125	753	1.5	131	3.5	5.76		
1.563	369	2.5	64	2.9	5.76		
0.781	183	2.8	32	7.3	5.67	5.64	2.1
0.391	89.9	2.1	16.2	5.1	5.55		
0.195	43.0	3.9	7.7	10.5	5.56		
0.098	19.4	10.3	3.6	11.7	5.44		
0.049	10.3	1.7	1.9	13.5	5.52		

Table 3. Accuracy and precision of the one-step RT-ddPCR method in quantifying the potency of a gene-repression vector (linearity)

Expected % relative potency	Measured % relative potency	Average measured % relative potency	% Recovery
150	162	164.0	109.3
	166		
125	132	130.5	104.4
	129		
75	72.9	72.8	97.0
	72.6		
50	48.9	49.5	99.0
	50.1		
		Accuracy (average % recovery)	98.0
		Precision (%CV)	1.4

RNase-free water and tested by both qRT-PCR with primer/probe set *SOD1* Set 1 in duplex with *Gapdh* and one-step RT-ddPCR with *SOD1* Set 2 in duplex with *Rpp30* (Figure 7A). The two methods show a linear correlation, with an R^2 value of 0.7248 and a slope of 1.071 (Figure 7B).

DISCUSSION

We developed a platform one-step RT-ddPCR method for quantifying AAV transgene expression and potency of a gene repression vector. We demonstrated key assay features in both *in vitro* and *in vivo* systems and compared this method to traditional two-step qRT-PCR methods and other orthogonal potency assays. This one-step RT-ddPCR method can serve as a framework for analytical and pharmacological assays in many upcoming gene therapy programs, both gene augmentation and repression. First, absolute quantification of target gene and a housekeeping reference gene in one duplex reaction allows for sample-specific internal control without standard curves. This absolute quantification nature enables inter-study data comparison with a simplified data output format. Second, the one-step RT and PCR process, coupled with automated droplet calculations, significantly shortens the hands-on manipulation in the workflow as well as the associated variability. In addition, as more academic and industry laboratories are using ddPCR to titer AAV vectors, it serves as a good complement to utilize the same platform technology for analytical and pharmacological assays to maintain consistency. With more automated ddPCR modules becoming commercially available, we anticipate an increase in adoption of this method in future analytics and pharmacological assays.

Although the one-step RT-ddPCR method has demonstrated high sensitivity, precision, linearity, as well as correlation to other methods, there are some considerations to keep in mind for certain applications of this method. First, the appropriate sample dilutions to reach the analytical range will need to be determined empirically, as this range is generally narrower in ddPCR than the qPCR counterpart. The linear range of qPCR is determined by the linearity and range of each standard curve, which could reach 5 copies to 1×10^{12} copies in one run. However, because of the physical limitation of 20,000 droplets per reaction, the linear range of ddPCR is below 1×10^5 copies per reaction. As a result, for samples with a wide range of mRNA expression levels, it is critical to dilute them within the linear range of the ddPCR-based method. This might require an additional spot-testing step prior to processing all test samples. In addition, because of the physical volume of droplets and the minimum number of droplets necessary for Poisson distribution analysis, the total reaction volume of ddPCR does not allow for further miniaturization. Therefore, there are only 96-well formats for ddPCR, unlike qPCR assays that could accommodate 384-well analysis for larger-scale experiments. Nonetheless, the RT-ddPCR method provides robust absolute quantification of target mRNA with a simplified workflow that can be further streamlined using laboratory automation systems.

In conclusion, a one-step RT-ddPCR method was developed and applied to both expression and potency assays for gene therapy drug development. Compared to orthogonal methods, this one-step RT-ddPCR platform method has several advantages, including

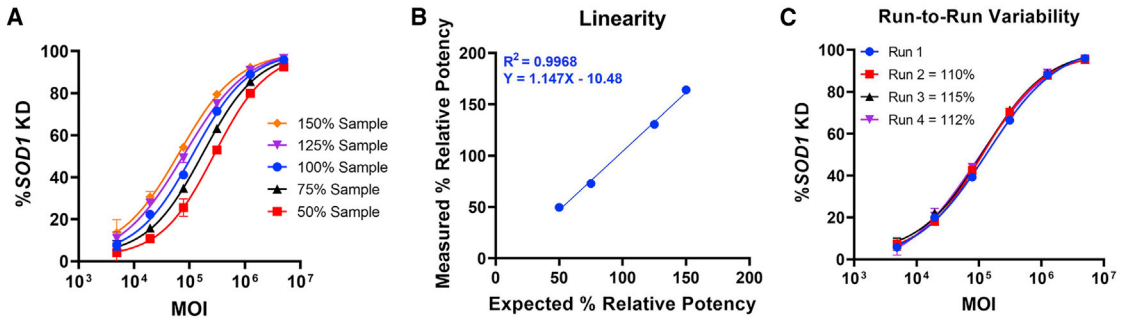

Figure 5. Preliminary qualification of the one-step RT-ddPCR method as a potency assay for AAV-RNAi vectors

HeLa cells were transduced with MOIs ranging from 1.03×10^4 vg/cell to 7.50×10^6 vg/cell. (A) Relative potency dose-response curves for vector preparations with expected potencies of 150%, 125%, 100%, 75%, and 50%. N = 6 independent replicates across 2 independent assays (N = 3 per assay). Error bars indicate standard deviation. (B) Average expected versus measured relative potencies to demonstrate linearity of the one-step RT-ddPCR potency assay. (C) Run-to-run variability of the same vector tested in the one-step RT-ddPCR potency assay with the first run set as the reference. N = 3 independent transduction replicates, each with individual RT-ddPCR result. Error bars indicate standard deviation.

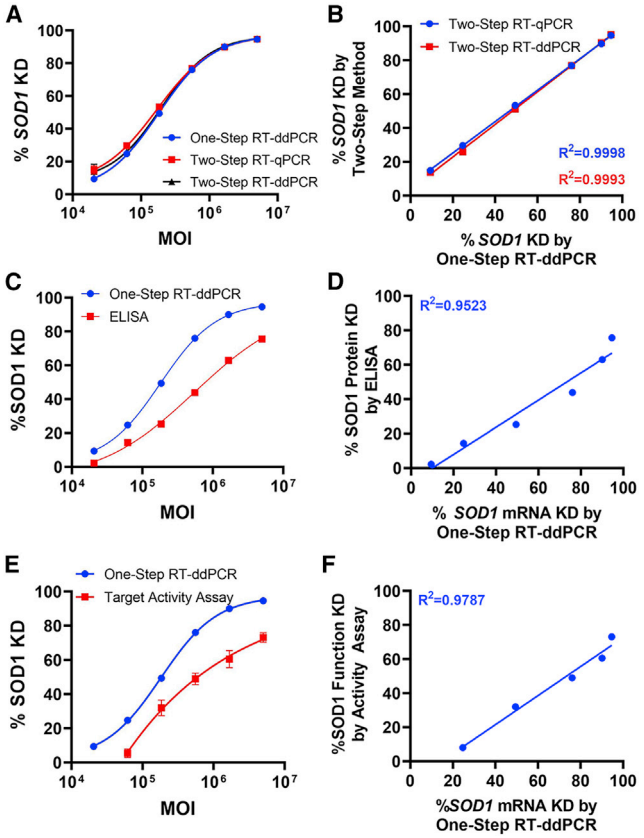

Figure 6. Correlation of gene repression vector *in vitro* potency quantified by one-step RT-ddPCR results and orthogonal methods

HeLa cells were transduced with MOIs ranging from 2.06 × 10^4 vg/cell to 5.00 × 10^6 vg/cell. (A) Quantification of AAV-mediated gene knockdown in relation to vector MOI measured by one-step RT-ddPCR, two-step RT-ddPCR, and two-step qRT-PCR. (B) Correlation of target average gene knockdown levels measured by one-step RT-ddPCR and two-step methods. (C) Quantification of AAV potency using target mRNA repression measured by one-step RT-ddPCR and protein reduction measured by ELISA. (D) Correlation of one-step RT-ddPCR and ELISA average results from (C). (E) Quantification of AAV potency using target mRNA repression measured by one-step RT-ddPCR and reduction of protein function measured by a SOD1 enzymatic activity assay. (F) Correlation of one-step RT-ddPCR and the SOD1 protein enzymatic activity average results from (E). N = 3 independent transduction replicates. Error bars indicate standard deviation. Some error bars are too small to be visible. Correlation plots utilize average of each measurement.

absolute quantification, simpler workflow, and greater robustness. As a result, more ddPCR-based methods will likely be implemented in the development of genetic medicines.

MATERIALS AND METHODS

AAV construction and production

All transgene plasmids for AAV production were cloned at Biogen using proprietary proviral vector backbones. Genes of interest were synthesized and subcloned into proviral vectors. AAV9-CAGGs-mCherry and AAVrh10-CAGGs-mCherry vectors were produced at UMASS Medical School Viral Vector Core (Worcester, MA, USA). AAV-RNAi vectors were produced at PackGene Biotech (Worcester, MA, USA). Titers of AAV vectors were quantified by ddPCR using transgene-specific primer/probe sets.

HeLa cell culture

HeLa cells were obtained from ATCC (CCL-2) and cultured in complete medium consisting of HyClone DMEM (Catalog #SH30022.02) supplemented with 10% fetal bovine serum (Sigma, Catalog #12103C).

Primers and probes

Custom primers and probe sets were designed with Primer Express 3.0.1 (Thermo Fisher Scientific), using both the TaqMan Quantification and TaqMan MGB Quantification settings. All custom primers and MGB probes were ordered from Thermo Fisher, and ZEN probes were ordered from Integrated DNA Technologies. When possible, PCR amplicons that span two different exons were chosen to ensure detection of cDNA from properly spliced mRNA and to mitigate detection of contaminating genomic DNA. Mouse *Gapdh* primer/probe set was ordered from Thermo Fisher (Catalog# 4352339E).

***SOD1* (Set 1)**

Forward Primer: 5′-TGGTGTGGCCGATGTGTCTA-3′

Reverse Primer: 5′-ATGATGCAATGGTCTCCTGAGA-3′

Probe: 5′-6FAM-TGAAGATTCTGTGATCTCA-MGB/NFQ-3′

***SOD1* (Set 2)**

Forward Primer: 5′-CTCTCAGGAGACCATTGCATCA-3′

Reverse Primer: 5′-CCTGTCTTTGTACTTTCTTCATTTCCA-3′

Probe: 5′-6FAM-CCGCACACT/ZEN/GGTGGTCCATGAAAA-IABkFQ-3′

mCherry

Forward Primer: 5′-TTGGACATCACCTCCCACAAC-3′

Reverse Primer: 5′-CCTCGGCGCGTTCGTA-3′

Probe: 5′-6FAM-ACTACACCATCGTGGAAC-MGB/NFQ-3′

WPRE

Forward Primer: 5′- TGTTGCTCCTTTTACGCTATGTG-3′

Reverse Primer: 5′ CGGGAAGCAATAGCATGATACA-3′

Probe: 5′- 6FAM-ATACGCTGCTTTAATGC-MGB/NFQ-3′

Human *RPP30*

Forward Primer: 5′-CACTGCAATGTTTTGAGAGCAACT-3′

Reverse Primer: 5′-TGGAAAAACTGCAACAACATCAT-3′

Probe: 5′-VIC-CTTCAAGGGCCCGGCT-MGB/NFQ-3′

Mouse *Rpp30*

Forward Primer: 5′-GCTGAGACGAGTCCTGAGT-3′

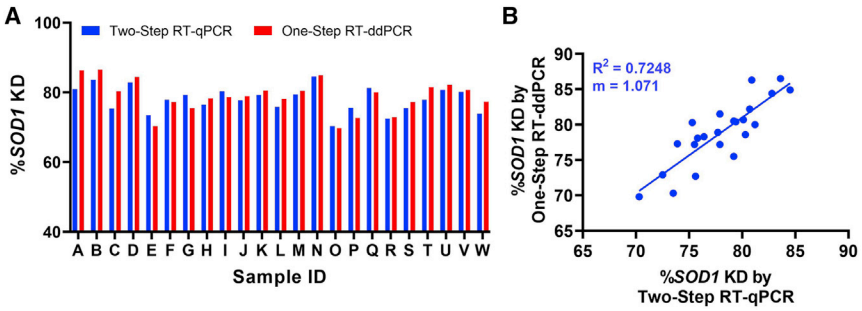

Figure 7. Correlation of gene repression vector *in vivo* potency quantified by one-step RT-ddPCR and two-step qRT-PCR

P0 SOD1-G93A mice were dosed at 8.0E+10 vg and 1.6E+11 vg by intracerebroventricular (ICV) injection. RNA was extracted from brain samples from each animal 20 weeks post-injection. (A) Percent knockdown of *SOD1* mRNA in SOD1-G93A mouse tissue samples measured by duplex one-step RT-ddPCR and qRT-PCR. Each animal sample was tested with 2 technical replicates in RT-ddPCR and 3 technical replicates in qRT-PCR. (B) Correlation of one-step RT-ddPCR and two-step qRT-PCR methods to measure average % knockdown of *SOD1* mRNA. Correlation plot utilizes average of each measurement.

Reverse Primer: 5′-CAGTGACTGATGAGCTACGA-3′

Probe: 5′-HEX-TCCACGCAC-ZEN-TGCCTCCTCCCC-IABkFQ-3′

One-step RT-ddPCR and analysis in HeLa cells

Cultured cells were seeded in a 96-well plate and incubated at 37°C overnight the day prior to transduction. Spent medium was aspirated off and replaced with AAV serial dilutions prepared in complete medium such that the desired MOI series was added to the plates. Transduced plates were incubated at 37°C for 48–72 h. After the incubation, medium was removed, and total RNA was extracted with the QIAGEN RNeasy Plus 96 Kit (Catalog #74192). Eluted RNA was diluted in nuclease-free water and combined with reagents from the One-Step RT-ddPCR Advanced Kit for Probes from Bio-Rad (Catalog #1864022) and two primer/probe sets each with final primer concentrations of 900 nM and probe concentrations of 250 nM. To measure vector expression, the primer/probe sets were *mCherry* or *WPRE* (FAM) and *RPP30* (VIC). To measure *SOD1* knockdown potency, the primer/probe sets were *SOD1* Set 2 (FAM) and *RPP30* (VIC). After the RT-ddPCR reaction setup, droplets were generated by the Automated Droplet Generator from Bio-Rad according to the manufacturer's instructions. After droplet generation, the droplets were subjected to endpoint PCR thermal cycling with the following protocol: 1 cycle of 48°C for 1 h; 1 cycle of 95°C for 10 min; 40 cycles of 94°C for 30 s, 60°C for 1 min; and 1 cycle of 98°C for 10 min followed by a 4°C hold. Droplet results were analyzed by QX200 Droplet Reader and QuantaSoft 1.7.4 software from Bio-Rad using the GEX analysis settings for FAM/VIC duplex reactions. This analysis automatically determines the relative expression levels or ratio of the target gene to the reference gene. To determine sensitivity for measuring vector expression, total RNA was first extracted from HeLa cells transduced with a high MOI of the AAV-RNAi vector and total RNA concentration was determined by NanoDrop. The RNA was serially diluted in RNase-free water and analyzed by one-step RT-ddPCR. Similarly, to determine sensitivity of the PCR targets used for potency assessment, naive HeLa RNA was extracted, measured by NanoDrop, serially diluted, and analyzed by one-step RT-ddPCR. Percent knockdown of *SOD1* for the potency assay was determined relative to uninfected control cells according to the following formula:

$$\%\ \mathrm{KD} = \frac{\frac{SOD1}{RPP30}\ \text{of infected cells}}{\frac{SOD1}{RPP30}\ \text{of control cells}} \times 100$$

Percent relative potencies were determined with the PLA 3.0 software by Stegmann Systems.

Non-human primate (NHP) study

This experiment was performed under IACUC compliance. Plasmid pSS305 was packaged into AAV9 or AAVrh10 using the triple transfection method in adherent HEK293 cells and purified by CsCl gradient ultracentrifugation. Vectors were buffer-exchanged and concentrated in final formulation buffer to a final concentration of 6.0×10^{13} VG/mL. Genome titer of each vector preparation was determined by ddPCR using the *WPRE* primer/probe set. 1.5×10^{14} vg of each AAV-CAGGs-mCherry was administered per animal in 2.5 mL injected into cerebrospinal fluid of cynomolgus monkeys via a bolus ICM injection. Twelve to fourteen days post-injection, animals were euthanized, and tissue samples were collected from different brain regions for biodistribution and vector expression analysis.

Gene expression in NHP samples by two-step qRT-PCR and analysis

NHP tissues were collected, treated with RNAlater, and frozen. Tissues were homogenized in Precellys tubes, and total RNA was extracted from each tissue lysate with the QIAGEN miRNeasy Mini Kit, including DNase I treatment. Concentration of total RNA was determined by NanoDrop. For qRT-PCR analysis, RNA was reverse transcribed into cDNA using MultiScribe Reverse Transcriptase from Thermo Fisher (Catalog# 4311235) and the *WPRE* reverse primer using the following thermal cycler protocol: 1 cycle of 25°C for 10 min; 1 cycle of 37°C for 120 min; and 1 cycle of 85°C for 5 min, followed by a 4°C hold. A standard curve for the qPCR was prepared by linearizing pSS305. The cDNA and linearized plasmid standard were combined with qPCR reaction mixture consisting of TaqMan Fast Advanced Master Mix and the *WPRE* primer/probe set. qPCR was performed on the QuantStudio 7 Flex Real Time PCR system with the following protocol: 1 cycle of 50°C for 2 min; 1 cycle of 95°C for 5 min; and 40 cycles of 95°C for 1 s, 60°C for 20 s. The number of copies of *WPRE* per 10 ng of starting RNA was reported.

Gene expression in NHP samples by one-step RT-ddPCR and analysis

For one-step RT-ddPCR analysis, total RNA samples that were extracted and analyzed by two-step qRT-PCR as described above were normalized to 20 ng/μL in RNase-free water based on NanoDrop results. Samples were then tested at two additional dilutions prepared in RNase-free water in duplex one-step RT-ddPCR with primer/probes for *mCherry* and *RPP30* using the RT-ddPCR protocol described above.

Two-step qRT-PCR and analysis of *SOD1* knockdown in HeLa cells

The same RNA eluate as described in the one-step RT-ddPCR method for cell-based assay was used to generate cDNA with the High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific (Catalog #4368814). The cDNA was combined with qPCR reaction mixtures consisting of TaqMan Universal Master Mix and the same primers and probes, at the same final concentrations, as described in the one-step RT-ddPCR setup. qPCR was performed on the CFX96 Touch Real-Time PCR Detection System with the following protocol: 1 cycle of 50°C for 2 min; 1 cycle of 95°C for 15 min; and 40 cycles of 95°C for 30 s, 60°C for 1 min. qPCR data were analyzed by the CFX Maestro software from Bio-Rad to generate Ct values. ΔΔCt analysis was performed to determine the percent *SOD1* knockdown relative to the control gene and the uninfected control cells.

Two-step RT-ddPCR and analysis of *SOD1* knockdown in HeLa cells

The same cDNA synthesized in the two-step qRT-PCR setup was used to prepare ddPCR reactions in 2× ddPCR Supermix for Probes (No dUTP) from Bio-Rad with the same final primer/probe concentrations. The same droplet generation and ddPCR workflow as described in the one-step RT-ddPCR method was used, including data analysis.

SOD1 ELISA and analysis

To measure knockdown of SOD1 protein, HeLa cells were transduced with the same MOI series as the one-step RT-ddPCR method in 150-mm dishes. After 72 h incubation at 37°C, cells were scraped from the plates, lysates were generated, and SOD1 protein was measured with a Human Superoxide Dismutase 1 ELISA Kit (Abcam). The manufacturer's instructions were followed for performing the ELISA. Percent knockdown of SOD1 was determined relative to the uninfected control cells in the same manner as in the one-step RT-ddPCR method.

SOD1 function assay and analysis

To measure knockdown of SOD1 activity, HeLa cells were transduced with the same MOI series as the one-step RT-ddPCR method in 6-well plates. After 72 h incubation at 37°C, cells were trypsinized, pelleted, and resuspended in 1× PBS. Homogenates were prepared by sonication, and SOD1 activity was measured with the Superoxide Dismutase (SOD) Colorimetric Activity Kit from Thermo Fisher Scientific (Catalog #EIASODC). The manufacturer's instructions were followed for performing the colorimetric assay. Percent knockdown of SOD1 was determined relative to the uninfected control cells in the same manner as in the one-step RT-ddPCR method.

SOD1 mouse experiment

This experiment was performed under IACUC compliance. P0 SOD1-G93A mice were dosed at 8.0E+10 VG and 1.6E+11 VG with the AAV-RNAi vectors by intracerebroventricular (ICV) injection to knock down the expression of *SOD1*. After a 20-week in-life portion, tissues were harvested and homogenized by SPEX SamplePrep 2010 Geno/Grinder. Total RNA was extracted with the QIAGEN miRNeasy Mini Kit and quantified by an Invitrogen Qubit Fluorometer.

SOD1 knockdown in mouse samples by qRT-PCR and analysis

For two-step qRT-PCR, cDNA was generated from total RNA with the High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific (Catalog #4368814). The cDNA was combined with qPCR reaction mixtures consisting of TaqMan Fast Advanced Master Mix with *SOD1* primer/probe Set 1 and mouse *Gapdh* primer/probe. qPCR was performed on the QuantStudio 12K Flex PCR system with the following protocol: 1 cycle of 50°C for 2 min; 1 cycle of 95°C for 2 min; and 40 cycles of 95°C for 1 s, 60°C for 20 s. ΔΔCt analysis was performed to determine the percent *SOD1* knockdown relative to *Gapdh*, and the data were normalized to vehicle-treated control samples.

SOD1 knockdown in mouse samples by RT-ddPCR and analysis

For one-step RT-ddPCR, the same total RNA extract from mouse tissues was diluted in RNase-free water and combined with one-step RT-ddPCR kit components as described above with the *SOD1* Set 2 primer/probe (FAM) and the mouse *Rpp30* primer/probe (HEX). The workflow for one-step RT-ddPCR as described above was followed. Percent knockdown of *SOD1* was determined relative to vehicle injected control animals according to the following formula:

$$\%\ \mathrm{KD} = \frac{\frac{SOD1}{Rpp30}\ \text{of AAV dosed animal}}{\frac{SOD1}{Rpp30}\ \text{of control animals}} \times 100$$

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AUTHOR CONTRIBUTIONS

P.C. and S.S. designed the experiments and wrote the paper. P.C., S.K.L., T.C., E.G., and S.C.L. performed the experiments. P.T. designed and oversaw the NHP study. All authors contributed to data analysis and interpretation.

DECLARATION OF INTERESTS

All authors were employees at Biogen at the time of the studies. The authors declare no competing interests.

REFERENCES

1. Wang, D., Tai, P.W.L., and Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. Nat. Rev. Drug Discov. *18*, 358–378.
2. Hudry, E., and Vandenberghe, L.H. (2019). Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality. Neuron *101*, 839–862.
3. Bolt, M.W., Whiteley, L.O., Lynch, J.L., Lauritzen, B., Fernández de Henestrosa, A.R., MacLachlan, T., Ulrich, P., Philip, B.K., Mahalingaiah, P.K., Fuller, C.L., and Compton, D.R. (2020). Nonclinical Studies that Support Viral Vector-Delivered Gene Therapies: An EFPIA Gene Therapy Working Group Perspective. Mol. Ther. Methods Clin. Dev. *19*, 89–98.
4. Lock, M., Alvira, M.R., Chen, S.J., and Wilson, J.M. (2014). Absolute determination of single-stranded and self-complementary adeno-associated viral vector genome titers by droplet digital PCR. Hum. Gene Ther. Methods *25*, 115–125.
5. Lock, M., McGorray, S., Auricchio, A., Ayuso, E., Beecham, E.J., Blouin-Tavel, V., Bosch, F., Bose, M., Byrne, B.J., Caton, T., et al. (2010). Characterization of a recombinant adeno-associated virus type 2 Reference Standard Material. Hum. Gene Ther. *21*, 1273–1285.
6. Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., et al. (2013). The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin. Chem. *59*, 892–902.
7. Dobnik, D., Kogovšek, P., Jakomin, T., Košir, N., Tušek Žnidarič, M., Leskovec, M., Kaminsky, S.M., Mostrom, J., Lee, H., and Ravnikar, M. (2019). Accurate Quantification and Characterization of Adeno-Associated Viral Vectors. Front. Microbiol. *10*, 1570.
8. Sanmiguel, J., Gao, G., and Vandenberghe, L.H. (2019). Quantitative and Digital Droplet-Based AAV Genome Titration. Methods Mol. Biol. *1950*, 51–83.
9. Suoranta, T., Laham-Karam, N., and Yla-Herttuala, S. (2021). Optimized Protocol for Accurate Titration of Adeno-Associated Virus Vectors. Hum. Gene Ther. https://doi.org/10.1089/hum.2020.318.
10. Furuta-Hanawa, B., Yamaguchi, T., and Uchida, E. (2019). Two-Dimensional Droplet Digital PCR as a Tool for Titration and Integrity Evaluation of Recombinant Adeno-Associated Viral Vectors. Hum. Gene Ther. Methods *30*, 127–136.
11. Strain, M.C., Lada, S.M., Luong, T., Rought, S.E., Gianella, S., Terry, V.H., Spina, C.A., Woelk, C.H., and Richman, D.D. (2013). Highly precise measurement of HIV DNA by droplet digital PCR. PLoS ONE *8*, e55943.
12. Hayden, R.T., Gu, Z., Ingersoll, J., Abdul-Ali, D., Shi, L., Pounds, S., and Caliendo, A.M. (2013). Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. J. Clin. Microbiol. *51*, 540–546.
13. Persson, S., Alm, E., Karlsson, M., Enkirch, T., Norder, H., Eriksson, R., Simonsson, M., and Ellström, P. (2021). A new assay for quantitative detection of hepatitis A virus. J. Virol. Methods *288*, 114010.
14. Warburton, L., Meniawy, T.M., Calapre, L., Pereira, M., McEvoy, A., Ziman, M., Gray, E.S., and Millward, M. (2020). Stopping targeted therapy for complete responders in advanced BRAF mutant melanoma. Sci. Rep. *10*, 18878.
15. Deiana, M., Mori, A., Piubelli, C., Scarso, S., Favarato, M., and Pomari, E. (2020). Assessment of the direct quantitation of SARS-CoV-2 by droplet digital PCR. Sci. Rep. *10*, 18764.
16. Muralidharan, K., Yekula, A., Small, J.L., Rosh, Z.S., Kang, K.M., Wang, L., Lau, S., Zheng, H., Lee, H., Bettegowda, C., et al. (2021). *TERT* Promoter Mutation Analysis for Blood-Based Diagnosis and Monitoring of Gliomas. Clin. Cancer Res. *27*, 169–178.
17. Rački, N., Morisset, D., Gutierrez-Aguirre, I., and Ravnikar, M. (2014). One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. Anal. Bioanal. Chem. *406*, 661–667.
18. Johnson, J.S., and Samulski, R.J. (2009). Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleolus. J. Virol. *83*, 2632–2644.
19. Nonnenmacher, M., and Weber, T. (2012). Intracellular transport of recombinant adeno-associated virus vectors. Gene Ther. *19*, 649–658.