Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600™ Droplet Digital™ PCR System

Blake Hummer[1] and Andrew M. Prantner[2]
[1] Bio-Rad Laboratories, Inc., 2560 55th St, Boulder, CO 80301
[2] Bio-Rad Laboratories, Inc., Digital Biology Center, 5731 W Las Positas Blvd, Pleasanton, CA 94588

Abstract

Precise and reproducible characterization of adeno-associated virus (AAV) vectors is critical for both preclinical and clinical applications of AAV-based gene therapy. An AAV vector genome may contain the inverted terminal repeat (ITR), an upstream control element, a downstream regulatory element, and a transgene. Because there are several potential target sequences within the AAV vector genome, multiple Droplet Digital PCR (ddPCR™) assays distributed throughout the genome can provide a more confident estimate of the encapsidated AAV vector genome concentration and genome identity. In this article, we demonstrate that high-resolution characterization of an AAV vector genome with multiple assays can be easily and reproducibly accomplished with the QX600 ddPCR System.

Introduction

Research into the use of recombinant AAV vectors for in vivo gene therapy for genetic diseases has increased substantially in recent years. Accurate and reproducible characterization of the encapsidated AAV vector genome is essential to compare both intra- and interlaboratory preclinical results as well as to ensure the safety and efficacy of clinical formulations. Two essential characteristics of an AAV vector genome are the physical titer (typically measured as the genome concentration) and the composition of the various modular DNA elements that comprise the complete genome (identity). Precise genome quantification is important because both preclinical and clinical dosing are based on genome concentration. In addition, verifying AAV vector identity is important in laboratories and manufacturing facilities that produce multiple AAV vectors with different combinations of regulatory elements and transgenes. These elements are commonly adjusted to optimize gene expression in target tissues for different diseases.

Because there are several potential target sequences within the AAV vector genome, multiple ddPCR assays distributed throughout the AAV vector genome can provide a more confident estimate of the encapsidated AAV vector genome concentration and genome identity (Prantner and Maar 2023). Therefore, this study characterized an AAV serotype 5 (AAV5) vector sample and a recombinant AAV serotype 2 (AAV2) reference standard stock solution (referred to hereafter as RSM2) using six-color Droplet Digital PCR with the QX600 System. The six-color analysis of the samples was performed on three separate days to evaluate the reproducibility of the concentration measurements.

Materials and Methods

AAV Vectors

Two AAV vectors — AAV5 and RSM2 — were used in these experiments. The AAV5 vector was a component from an AAV GFP Testing Kit (Charles River Laboratories, catalog #CT0002). This viral vector genome, which is packaged into an AAV capsid, has ITRs from AAV2 (AAV ITR-2) flanking an expression cassette containing enhanced GFP (eGFP), a cytomegalovirus (CMV) promoter/enhancer, and an SV40 poly(A) signal sequence.

For RSM2, we used Recombinant Adeno-associated virus 2 (American Type Culture Collection [ATCC], #VR-1616), which is sold as a reference standard stock. This vector genome has an expression cassette containing humanized GFP (*hGFP*), a synthetic CMV/chicken β-actin (CMB) promotor, a SV40 poly(A) signal sequence, a neomycin/kanamycin resistance gene (*NeoR/KanR*), a human bovine growth hormone (*bGH*) poly(A) signal sequence, and two terminal AAV ITR-2 sequences.

ddPCR Assays

Published primer and probe sequences were used for detection of enhanced green fluorescent protein (*eGFP*) (Lock et al. 2014). The assay using the Lock et al. primers and probes is referred to hereafter as *GFP-L* to distinguish it from a second *eGFP* assay targeting a different region of the open reading frame. Primers and a PrimeTime ZEN Double-Quenched Probe were synthesized for *GFP-L* by Integrated DNA Technologies. Stock solutions were made by resuspending the primers and probes with

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DNA Suspension Buffer (Teknova, #T0223) to 100 µM. Working solutions of 20x assays (18 µM each primer and 5 µM probe) in DNA Suspension Buffer were prepared in Eppendorf DNA LoBind Microcentrifuge Tubes (Fisher Scientific, #13-698-791) for a final 1x reaction concentration of 900 nM for each primer and 250 nM for the probe.

All other assays are available from Bio-Rad as ddPCR Expert Design Assays. Assay targets for AAV5 were: AAV ITR-2 (FAM; assay ID: dEXD15274642), *GFP-L* (HEX; see previous paragraph), CMV promoter (Cy5; dEXD35030798), *eGFP* (Cy5.5; dEXD46876944), CMV enhancer (ROX; dEXD81376879), and SV40 poly(A) (ATTO 590; dEXD49426647). Assay targets for RSM2 were: AAV ITR-2 (FAM; dEXD15274642), *hGFP* (HEX; dEXD96535526), CMV enhancer (Cy5; dEXD16393963), *bGH* poly(A) (Cy5.5; dEXD69882860), *NeoR/KanR* (ROX; dEXD76674098), and SV40 poly(A) (ATTO 590; dEXD49426647).

AAV Vector Preparation

To remove any unencapsidated DNA in the AAV5 and RSM2 samples, samples were first digested with DNase I (New England Biolabs Inc., #M0303L) in 50 µl reactions that contained 5 µl viral vector, 5 µl of 10x DNase I Reaction Buffer (NEB, #B0303S), 5 µl of Poloxamer 188 Non-Ionic Surfactant, 100x (1% Pluronic-F68; Thermo Fisher Scientific, #24040032), 5 µl of DNase I, and 30 µl of nuclease-free water (not treated with diethyl pyrocarbonate [DEPC]). Reactions were incubated at 37°C for 30 min and then serially diluted tenfold using poly(A)+ buffer in DNA LoBind Tubes. Poly(A)+ buffer consists of: 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 100 µg/ml Poly(A) (MilliporeSigma #10108626001), and 0.01% Pluronic F-68. Aliquots of samples in the ddPCR concentration range were then incubated in PCR tubes with a C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (Bio-Rad, #1851197) at 95°C for 10 min to thermally lyse the capsids and then rapidly cooled at a rate of 3°C/sec to 4°C, prior to being used as the template for ddPCR reactions. A more comprehensive description of the protocol can be found in: Measuring Adeno-Associated Virus (AAV) Vector Genome Titer Using Droplet Digital PCR Protocol (bulletin 7407).

Droplet Digital PCR and Data Analysis

Each 20 µl six-color reaction contained 5 µl of ddPCR Multiplex Supermix (Bio-Rad, #12005911), 1 µl of 20x assay mix for each of the six targets, 1 µl of AAV vector DNA, 5 U (0.25 µl) of MspI (NEB # R0106S), and water. Four reaction volumes (two technical replicates for partitioning plus additional sample volume to account for liquid loss to solid surfaces and for pipetting convenience due to the small volume of MspI) were prepared for each AAV vector. Samples were pipet-mixed, thoroughly vortexed, pulsed in a microcentrifuge, and droplets were generated with a droplet generator. Droplets were then transferred to ddPCR 96-Well Plates (Bio-Rad, #12001925), sealed with pierceable metal PCR Plate Heat Seals (Bio-Rad #1814040), and thermal cycled in a C1000 Touch Thermal Cycler using a ramp rate of 2°C/sec with the following parameters: 95°C for 10 min, 40 cycles of 94°C for 30 sec, then 55°C for 60 sec, 98°C for 10 min, and a final hold at 4°C. The fluorescence signal from the droplets was read using a QX600 Droplet Digital PCR System (Bio-Rad, #17007769). Data from the two technical replicates for each sample were merged prior to data analysis. For merged wells, the reported concentrations were determined by Poisson analysis of the positive and negative droplets and the 95% confidence interval for the total error. These calculations were performed by QX Manager Software, Standard Edition (version 2.1).

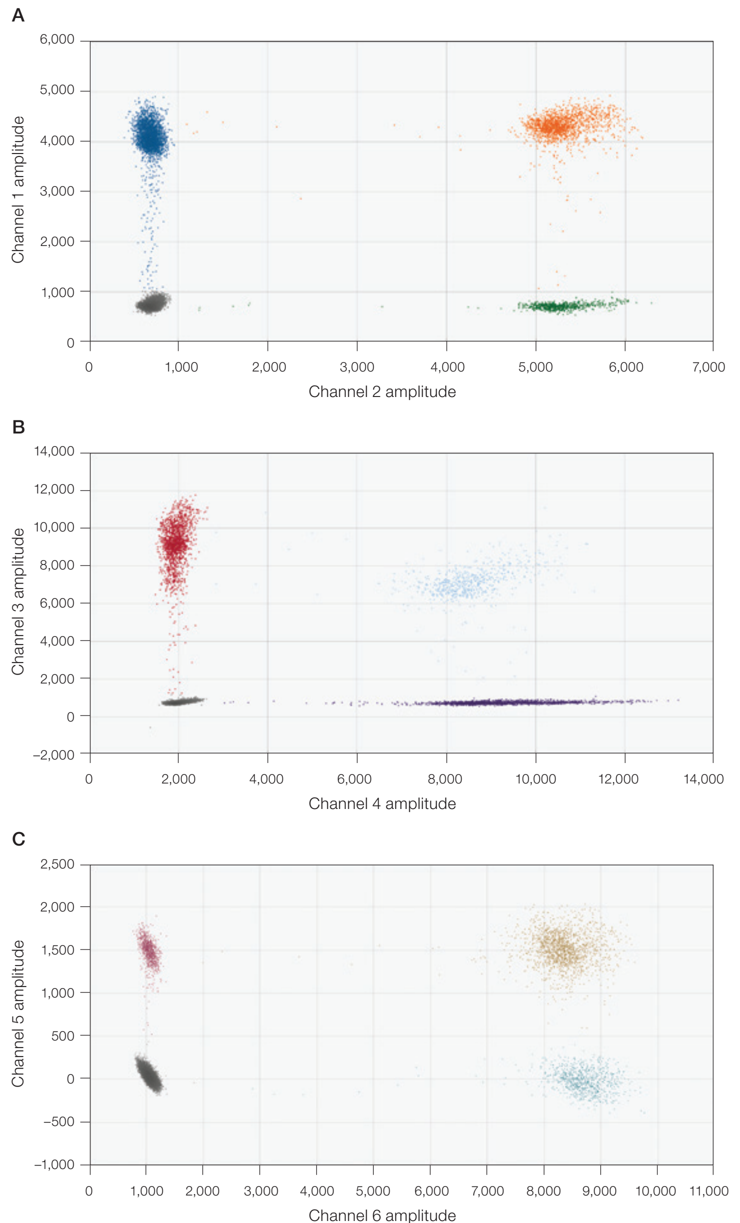

Fig. 1. Two-dimensional fluorescence intensity plots for 6-plex analysis of RSM2. Representative plots for a six-color experiment using RSM2 vector DNA as the template. **A**, data for AAV ITR-2 (FAM; blue) and humanized GFP (*hGFP*) (HEX; green). The orange cluster represents droplets positive for both targets, whereas the gray cluster represents droplets negative for both. **B**, data for CMV enhancer (Cy5; red) and bovine growth hormone (*bGH*) poly(A) (Cy5.5; purple). The light blue cluster represents droplets positive for both targets, whereas the gray cluster represents droplets negative for both. **C**, data for neomycin/kanamycin resistance (*NeoR/KanR*) (ROX; dark red) and SV40 poly(A) (ATTO 590; cyan). The gold cluster represents droplets positive for both targets, whereas the gray cluster represents droplets negative for both. Tilt correction was applied.

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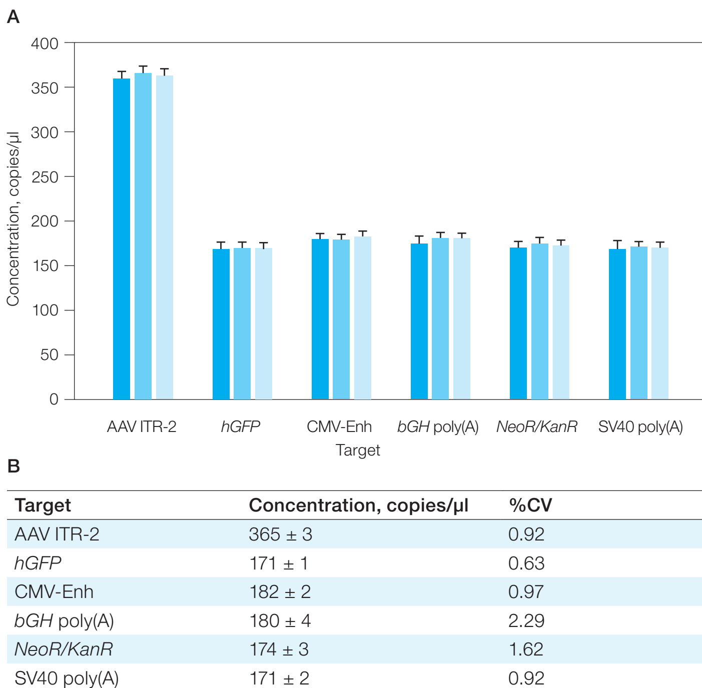
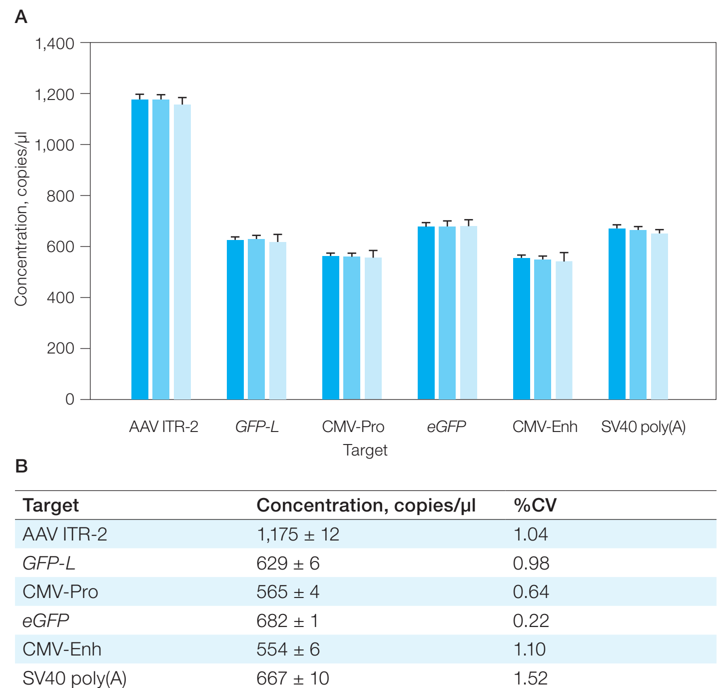


Target	Concentration, copies/µl	%CV
AAV ITR-2	365 ± 3	0.92
hGFP	171 ± 1	0.63
CMV-Enh	182 ± 2	0.97
bGH poly(A)	180 ± 4	2.29
NeoR/KanR	174 ± 3	1.62
SV40 poly(A)	171 ± 2	0.92

Fig. 2. Reproducibility of RSM2 ddPCR analysis. The concentration of six targets in an RSM2 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average and standard deviation for the three concentration measurements (given in copies/µl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer.



Target	Concentration, copies/µl	%CV
AAV ITR-2	1,175 ± 12	1.04
GFP-L	629 ± 6	0.98
CMV-Pro	565 ± 4	0.64
eGFP	682 ± 1	0.22
CMV-Enh	554 ± 6	1.10
SV40 poly(A)	667 ± 10	1.52

Fig. 3. Reproducibility of AAV5 ddPCR analysis. The concentration of six targets in an AAV5 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average standard deviation for the three concentration measurements (given in copies/µl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer; CMV-Pro, CMV promoter.

For repeatability analysis, individual aliquots were removed each day from the diluted AAV vector samples, thermally lysed, and analyzed by six-color Droplet Digital PCR. The concentration of target DNA in each assay, which was determined on three different days, was averaged and the standard deviation was determined to calculate the coefficient of variation (%CV).

Results

Representative fluorescence intensity plots for 6-plex ddPCR analysis of RSM2 are shown in Figure 1. All six targets were successfully detected, with clear separation between positive and negative droplets for each target. To assess the reproducibility of this analysis, individual aliquots from an RSM2 sample were analyzed with a 6-plex ddPCR assay on three different days. The individual concentrations of the six targets measured each day are shown in Figure 2A. The concentration data for the three days was used to calculate the average concentration, standard deviation, and %CV (Figure 2B). There was excellent agreement between the concentration values obtained on separate days, as indicated by a %CV of less than 2% for all six assays.

An analogous 6-plex experiment was performed for an AAV5 vector, in which individual aliquots were analyzed with a 6-plex ddPCR experiment across three different days. The concentrations of the six targets obtained each day are shown in Figure 3A. The concentration data for the three days were used to calculate the average concentration, standard deviation, and %CV (Figure 3B). As with the RSM2 sample, there was excellent agreement between the concentration values obtained on separate days for the AAV5 vector, which is indicated by a %CV of less than 2.5% for all six assays.

Conclusion

These data demonstrate that six DNA targets can be simultaneously evaluated by Droplet Digital PCR using the QX600 System to comprehensively characterize the identity of an AAV vector sample. High-resolution ddPCR analysis of AAV vectors using six assays distributed throughout the AAV vector genome can provide confidence in the concentration and identity of an AAV vector sample prior to preclinical or clinical applications. In addition, the individual concentration measurements, when compared on three different days, were highly reproducible, enabling their use in evaluating AAV manufacturing or formulation strategies.

References

Lock M et al. (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.

Prantner A and Maar D (2023). Genome concentration, characterization, and integrity analysis of recombinant adeno-associated viral vectors using Droplet Digital PCR. PLoS One 18, e0280242.

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