Digital PCR

Characterization of AAV Genomic Titer on the Applied Biosystems™ QuantStudio™ Absolute Q™ dPCR System

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Abstract

Purpose: Investigate the use of the QuantStudio Absolute Q digital PCR (dPCR) system for determining accurate recombinant adeno-associated virus (rAAV) genome titer.

Methods: Digital PCR (dPCR) using TaqMan chemistry and commercially available rAAV particles treated with heat-lysis.

Results: Consistent quantitation of genomic copies observed over several experiments without the need for a standard curve and optimized using buffer containing surfactant and HEK293 cell lysate.

Introduction

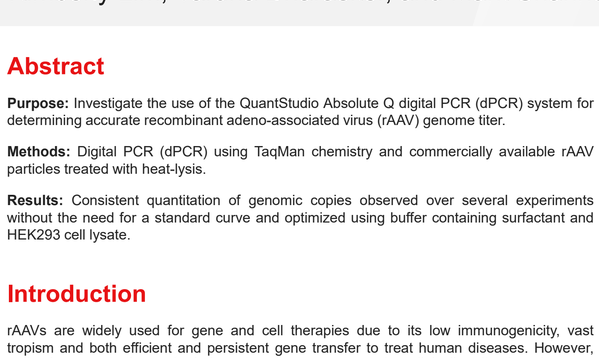
rAAVs are widely used for gene and cell therapies due to its low immunogenicity, vast tropism and both efficient and persistent gene transfer to treat human diseases. However, optimal concentrations of viral vectors are necessary for clinically effective dosages. Accurate, precise, and consistent viral titer measurements are required throughout routine quality control processes for rAAV vector preparations.

Quantification of genomic copies (GC/mL) is important for characterizing rAAV. While quantitative PCR (qPCR) is considered the gold standard method for determining the genomic titer, this approach requires the use of standard curves and is thus more susceptible to variation from run to run. dPCR provides significant advantages for analytical assays necessary for viral vector production and characterization without the need for a standard curve. dPCR is also less sensitive to contaminants that affect amplification, including those present in solutions used during the development of AAV-mediated gene therapies.

In this work, we utilize the QuantStudio Absolute Q dPCR system to investigate the platform's use for determining accurate rAAV genome titer. Using commercially available purified AAV particles, we found consistent quantitation of genomic copies across several AAV serotypes when multiplexing assays that targeted different regions of the AAV genome. Results were obtained without the need for a standard curve, nor reference standard material. We also observed that while qPCR reactions are highly affected by the presence of HEK293 cell lysate, dPCR reactions are more tolerant to the presence of cell lysate and even less affected when cell lysate is diluted in TE buffer.

Workflow

Figure 1. Process map for the treatment of AAV samples.



Samples are diluted in buffer containing surfactant, and within the dynamic range of the QuantStudio Absolute Q dPCR system.

Samples are heated to 95˚C for 5 minutes on a thermocycler to break open viral capsids and release DNA.

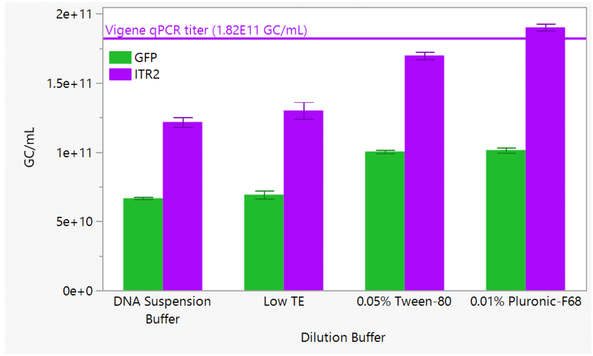
9µL reaction loading onto the QuantStudio Absolute Q MAP16 plate takes less than 5 minutes, while the dPCR run and analysis takes approximately 90 minutes.

Results

AAV Particle Treatment

Addition of surfactant was found to be required for optimal detection and quantitation of AAV genomic targets. qPCR titer from Vigene (catalog #: RS-AAV2-FL) was reported on the certificate of analysis (1.82E11 GC/mL). The presence of surfactant may assist in viral particle lysis and prevent viral particles from clumping together and attaching to plastic walls of dilution tubes[1].

Figure 2. Dilution buffer and AAV particle treatment

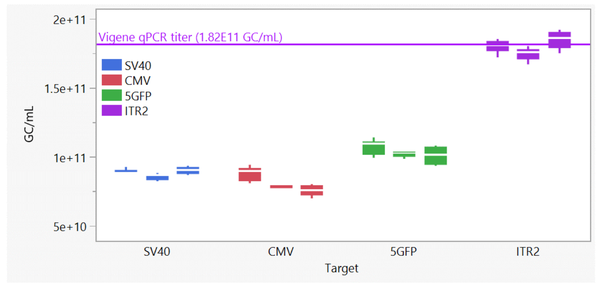


Comparison of AAV2 particles prepared using various dilution buffers and tested for genomic titer using duplexed of a custom GFP assay and the Absolute Q AAV ITR-2 assay (A52740) on dPCR. GC/mL was calculated using copy/uL results obtained from the QuantStudio Absolute Q dPCR system and multiplied by dilution factors needed to create the sample. N=4 per sample.

Reproducibility of AAV genomic titer

Based on the previous results, 0.05% Tween-80 buffer was used for viral dilutions in subsequent experiments. Consistent genomic titer was found across three independent experiments testing AAV2 particles and four genomic targets: SV40, CMV promoter, GFP and ITR2.

Figure 3. Multiplex dPCR reaction testing AAV2 particles

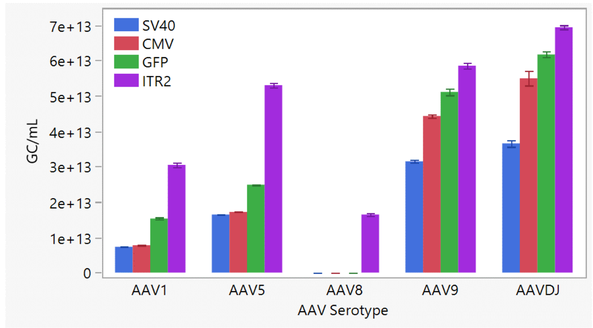


AAV2 particles tested on the Absolute Q dPCR platform across three independent runs using TaqMan PCR assays targeting SV40 (ABY), CMV promoter (JUN), 5' region of GFP (FAM) and ITR2 (VIC). All assays were multiplexed in the same dPCR reaction. N=6 for all samples.

AAV serotypes genomic titer

Multiplex reactions of custom dPCR assays with ITR2 viral titer assay were tested against several types of AAV serotypes to demonstrate compatibility on the Absolute Q dPCR system. Serotypes were purchased as part of a kit containing different serotypes with the same genome (AMSBio catalog #: CT0002). Notably, SV40, CMV and GFP were not detected in AAV8 samples.

Figure 4. Genomic titer of different AAV serotypes through multiplex dPCR.

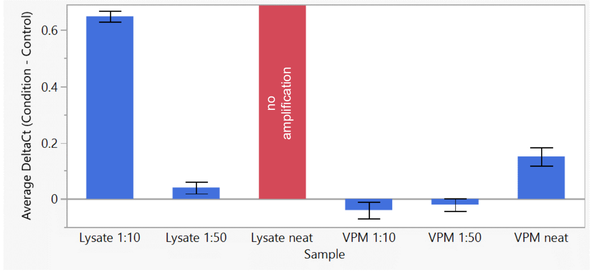


Various serotypes of commercially available AAV particles containing the same genomic plasmid were tested for genomic titer using a multiplex of four Taqman PCR assays: SV40 (ABY), CMV promoter (JUN), 5' region of GFP (FAM) and ITR2 (VIC). N=4 for all samples.

Cell lysate study comparison between qPCR and dPCR

To determine if the Absolute Q assay and system are less sensitive to contaminants found in lysates, performance of DNA control samples containing various concentrations of lysate were tested and compared between qPCR and dPCR. Cell lysate was collected from HEK293 cells lysed using Gibco AAV-MAX Lysis Buffer (catalog #: A50520). DNA control (DNA string from GeneArt) was spiked into various dilutions of cell lysate or empty Gibco Viral Production Media (catalog #: A4817901) and tested using qPCR. Complete inhibition was observed in samples containing undiluted lysate (neat) while slight changes in cycle Ct were observed in all other samples with compared to samples containing DNA control diluted in TE buffer only.

Figure 5. Cell lysate affects qPCR reaction

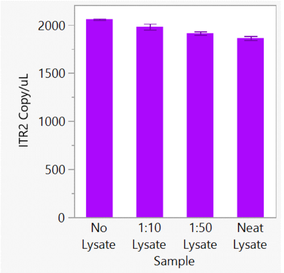


Undiluted lysate (neat) and lysate diluted 1:10 or 1:50 with TE buffer were tested using qPCR. One concentration of DNA control was tested across all samples. Average ΔCt was calculated by subtracting control sample (DNA control diluted in TE buffer only). ITR2 assay was tested in singleplex. N=4 For all samples. VPM – viral production media.

dPCR reaction resistant to cell lysate

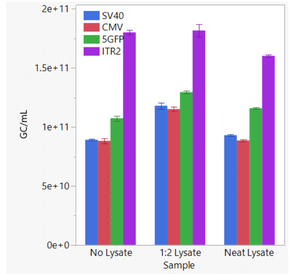
DNA control (Figure 6A) and AAV2 particles (Figure 6B) were tested in the presence of cell lysate using dPCR. Slight changes in copy/uL results were observed in samples containing different dilutions of lysate,

Figure 6A. DNA control with cell lysate tested on dPCR



ITR2 was tested in singleplex in dPCR. N=2 for each sample.

Figure 6B. AAV2 particles with cell lysate tested on dPCR



SV40, CMV GFP and ITR2 were tested in multiplex on dPCR. N=3 for each sample.

Conclusions

In summary, we showcase several advantageous features of the QuantStudio Absolute Q digital PCR system for biopharma and gene therapy research:

1) simple and fast workflow
2) consistent quantification of AAV genomic titer without the need for standard curves
3) higher resistance to PCR inhibition due to host cell lysis

References

1) Lock, M.; Alvira, M.R.; Chen, S.J.; Wilson, J.M. Absolute Determination of Single-Stranded and Self-Complementary Adeno-Associated Viral Vector Genome Titers by Droplet Digital PCR. *Hum. Gene Ther. Methods* **2014**, 25, 115–125.

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