Digital PCR

Measuring rAAV Genomic Titer, Viral Particle Titer and Full/Empty Capsid Ratio on the Applied Biosystems™ QuantStudio™ Absolute Q™ dPCR System

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Introduction

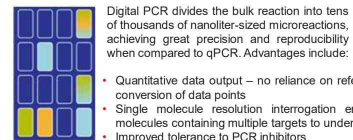
Recombinant adeno-associated viruses (rAAV) are widely used for human gene and cell therapies due to their low immunogenicity, vast tropism and efficient and persistent gene transfer properties. Accurate and consistent viral titer measurements are needed throughout all phases of rAAV vector production from early stages of developing AAV expression systems to production scale-up and final product release.

Quantification of genomic titer (GC/mL) and viral particle titer (vp/mL) as well as the ratio of full to empty capsids are important rAAV attributes. However, quantification is not done in a harmonized manner. Other limited methods can measure single rAAV characteristics: qPCR measures genomic titer while viral particle titer is measured by ELISA. Full/empty ratio can be inferred from the combination of qPCR and ELISA data or measured independently by electron microscopy, analytical ultracentrifugation, or high-pressure liquid chromatography.

Proximity ligation assay (PLATM) is an immunoassay approach that leverages PCR amplification to quantify and enable both full and empty capsid studies on a single qPCR platform. However, this approach requires the use of standard curves and reference standards for determining particle and genome titers. As such, the method is more susceptible to inter-assay variation as well as variation due to heterogeneity of genomic content in full and partial capsids.

Digital PCR (dPCR) which enables absolute quantification of nucleic acid targets without standard curves, would remove this variation. Here, our proof-of-concept experiments demonstrates the approach of quantifying genome and particle titer in a multiplex reaction on the Absolute Q dPCR system (Figure 1). The Absolute Q also enables linkage analysis to be performed along with capsid quantification, which may provide a more comprehensive view of full and partially-filled capsids. Proof of principles to demonstrate this effort with a 4-plex assay will also be discussed.

Absolute Q Digital PCR

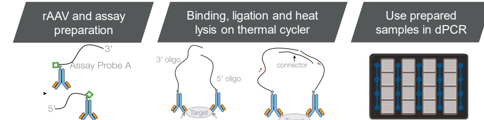


Digital PCR divides the bulk reaction into tens of thousands of nanoliter-sized microreactions, achieving great precision and reproducibility when compared to qPCR. Advantages include:

- Quantitative data output – no reliance on references or standards for conversion of data points
- Single molecule resolution interrogation enables quantification of molecules containing multiple targets to understand molecular integrity
- Improved tolerance to PCR inhibitors

Workflow

Figure 1. Digital PCR workflow for protein quantification with the Absolute Q



rAAV are diluted in buffer containing surfactant within the dynamic range of the QuantStudio Absolute Q dPCR system. PLA probes are prepared by attaching biotinylated AAVX antibodies (catalog #: 7103522500) to proprietary streptavidin-oligonucleotides conjugates. Probes are then combined with AAV samples.

Samples are incubated to 37˚C for 4 hours on a thermal cycler to allow probes to bind to viral epitopes. Ligase is then added along with connector oligo, dPCR assay and master mix. Samples are heated to 95˚C for 5 minutes to inactivate ligase, lyse 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-PLA on dPCR

Genome titer, viral particle titer and full/empty capsid ratio of AAV2 (ATCC, catalog #: VR-1616) and AAV6 (Vector Biolabs, catalog #: 7008) can be measured on the Absolute Q platform in a duplex reaction. High sample concentration in AAV6 Dilution 4 may have oversaturated the dPCR signal, thus lowering viral particle titer.

Figure 2A. dPCR result for two rAAV serotypes

Virus	5-fold Dilution	PLA Conc. Copies/µL	ITR2 Conc. Copies/µL	Average Estimated dPCR Viral Particle Titer (vp/mL)	Average Estimated dPCR Genomic Titer (GC/mL)	Average % full capsids (Genomic titer/ VP titer)
AAV2 (VR1616)	Dilution 3	292	42	3.87E+12	1.58E+12	40.7%
	Dilution 4	1236	197	3.30E+12	1.47E+12	45.2%
	Dilution 5	5888	861	2.30E+12	1.43E+12	62.5%
AAV6 (VBL7008)	Dilution 2	1239	505	1.83E+11	2.63E+10	14.3%
	Dilution 3	5279	2352	1.55E+11	2.46E+10	15.8%
	Dilution 4	18369	11443	1.47E+11	2.15E+10	15.1%

Duplex reaction of proximity-ligation assay and Absolute Q AAV ITR2 assay (A52740) on dPCR. VP/mL and GC/mL were calculated using copy/µL results obtained from the QuantStudio Absolute Q dPCR system and multiplied by dilution factors needed to create the sample % full particles was calculated by dividing genomic titer by viral particle titer. N=4 per dilution.

Figure 2B. qPCR result for two rAAV serotypes

Virus	Average Estimated qPCR Viral Particle Titer (vp/mL)	Average Estimated qPCR Genomic Titer (GC/mL)	Average % full particles (Genomic Titer/ VP Titer)
AAV2 (VR1616)	6.66E+12	2.45E+12	36.9%
AAV6 (VBL7008)	4.46E+11	4.88E+10	10.9%

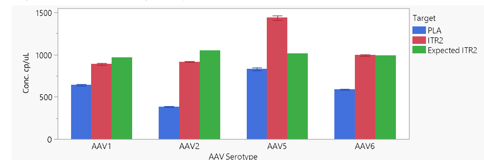
Duplex reaction of proximity-ligation assay and Absolute Q AAV ITR2 assay (A52740) on dPCR. VP/mL and VG/mL were derived from a standard curve of well-characterized AAV reference standards from Vigene (RS-AAV2-FL and RS-AAV6-FL). % full particles was calculated by dividing genomic titer by viral particle titer.

The full/empty capsid ratio derived from dPCR for the two AAV serotypes tested aligned closely with the same measurement derived from qPCR standard curves. Although the estimated viral particle and genomic titers were higher in qPCR compared to dPCR, qPCR titers may be overestimated due to inaccurate standard curve or reference material.

Various AAV serotypes are compatible with PLA and TaqMan™ assays

AAV reference standards from Vigene (AAV2: RS-AAV2-FL, AAV6: RS-AAV6-FL) and Amsbio (AAV1: CV10003, AAV5: CV10005) were evaluated using PLA and several genomic assays. AAVX antibodies may have varying binding efficiencies across serotypes and may require additional optimization. ITR2 results were within 15% expected concentration from the CoA for AAV1, 2, and 6. Greater estimation of ITR2 concentration over PLA or viral particle concentration may be caused by detection of two copies of ITR2 as well as inefficient ssDNA encapsidation. Interrogating multiple genomic targets can create a clearer picture of the different DNA species encapsidated in these reference standards.

Figure 3. AAV-PLA using serotypes 1, 2, 5 and 6

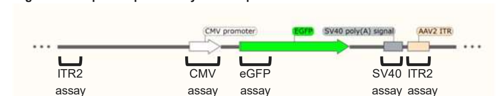


CoA genomic titers from manufacturers were determined using qPCR and ITR-specific primers. Duplex reaction consisted of PLA assay and Absolute Q AAV ITR2 assay, N=4 for each bar.

Evaluating AAV Genome Integrity on dPCR

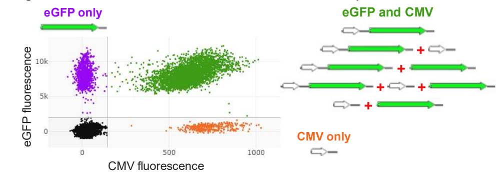
Interrogating different AAV genomic targets with multiplex assays can help to show the quality of genome integrity in full or partial AAV capsids[1] (Figure 4A). Absolute Q dPCR divides DNA molecules into thousands of microchambers, which may allow single-molecule resolution for evaluating genomic composition of AAV. However, linked targets are undistinguishable from two targets co-occupying a microchamber by chance (Figure 4B). Linkage analysis calculates for the number of double-positive microchambers not caused by chance double-occupancy[2]. % linked results are not affected by DNA input concentration.

Figure 4A. Map of TaqMan assays in AAV plasmid



TaqMan assays used for testing AAV particles include the Absolute Q AAV ITR2 assay (A52740) and three custom TaqMan assays targeting enhanced green fluorescent protein (eGFP) in FAM, simian virus 40 (SV40) polyA signal in ABY and cytomegalovirus promoter (CMV) in JUN. DNA sequence provided by Vigene for AAV2 and AAV6.

Figure 4B. DNA combinations detected in eGFP-CMV double-positive microchambers



Five possible combinations of DNA molecules can produce double-fluorescence for eGFP and CMV. 2D dot plot from multiplex linkage analysis in Figure 5.

AAV2 and AAV6 were tested using the four genomic assays on Absolute Q dPCR. Samples were tested at three concentrations (ITR2 lambda: 0.7, 0.2, 0.05). % linkage was calculated as described in Regan, 2015[2]. Results indicate variable linkage between different genomic targets, which could be caused by presence of partial AAV capsids and bias towards the 3' end of the ssDNA strand.

Figure 5. Linkage analysis of AAV2 and AAV6

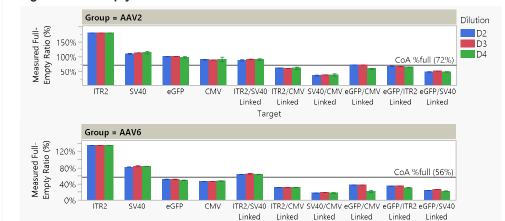
Targets				% Linkage		
ITR2	CMV	eGFP	SV40	Control	AAV2	AAV6
				96%	78%	77%
				98%	53%	45%
				94%	52%	43%
				95%	51%	44%
				95%	66%	66%
				92%	43%	36%

AAV2 and AAV6 particles tested on the Absolute Q dPCR platform using TaqMan assays targeting ITR2 (VIC), SV40 (ABY), eGFP (FAM) and CMV promoter (JUN). Control DNA is DNA string containing sequences for all four targets. All assays were multiplexed in the same dPCR reaction. N=4 for control sample, N=6 for AAV2 and 6 across three concentrations. Red boxes indicate targets interrogated for linkage.

Calculating full/empty capsid ratio using multiplex data on dPCR

The absolute concentration of linked molecules was calculated from results in Figure 5 and divided by PLA results of AAV2 and AAV6 from Figure 3 to calculate for full/empty capsid ratio, which was found to be highly dependent on the chosen genomic target(s (Figure 6). As ITR2 was observed to have higher copies/µL compared to PLA copies/µL in Figure 3, a full/empty ratio above 100% was expected. By using linkage analysis, interrogating several AAV genomic targets can allow for improved estimation of functional genomes.

Figure 6. Full-empty ratios of AAV2 and AAV6



AAV2 and AAV6 particles tested on the Absolute Q dPCR platform using TaqMan assays targeting ITR2 (VIC), SV40 (ABY), eGFP (FAM) and CMV promoter (JUN), multiplexed in the same dPCR reaction. Full/empty ratio was calculated using results from a single target's copies/µL result or derived copies/µL from linkage analysis from Figure 5, then divided by PLA results from Figure 3. CoA %full determined by transmission electron microscopy. N=2 for each bar.

Conclusions

In summary, we demonstrate the feasibility of determining AAV genome titer, viral particle titer and full/empty capsid ratio in a single duplexed dPCR reaction as well as investigating AAV genome integrity using linkage analysis from multiplex dPCR reactions. In this work, we used commercially available purified AAV particles as the test samples. Further work is needed to understand the impact of using unpurified or partially purified samples on multiplex assay performance. Also, the differences in AAVX antibody affinity to different serotypes should be investigated to determine potential impact on sensitivity and precision. This work demonstrates the ability of the Absolute Q digital PCR system to improve and further simplify AAV characterization, benefiting cell and gene therapy efforts from early development stages to production scale-up and final product release. Advantages of this method include:

- analyzing genome and particle titer measures on one platform
- generating absolute quantification data without the need for standard curves
- evaluating genome integrity using multiplex reactions

References

1. Furuta-Hanawa B, Yamaguchi T, Uchida E. Two-Dimensional Droplet Digital PCR as a Tool for Titration and Integrity Evaluation of Recombinant Adeno-Associated Viral Vectors. *Hum Gene Ther Methods*. 2019;30(4):127-136. doi:10.1089/hgtb.2019.031

2. Regan JF, et al. (2015) A Rapid Molecular Approach for Chromosomal Phasing. *PLoS ONE*. 10(3): e0118270. doi:10.1371/journal.pone.0118270.

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