Application Note

A duplex assay for quantification and qualification of adeno-associated virus (AAV) using the QIAcuity® Digital PCR System

Carey Carver, Yotam Blech-Hermoni, James Qin

Applications Lab, QIAGEN Sciences, USA

Introduction

Adeno-associated virus (AAV) is a widely used viral vector in gene therapy applications. Accurate quantitation and characterization of AAV are critical for precise dosing during clinal studies or patient care. qPCR is a widely used method for AAV quantitation due to its sensitivity and ease of use.

When performing absolute quantitation with qPCR, DNA standards of known concentration are needed to plot a standard curve which is then used to extrapolate the titer of the unknown sample. To ensure accuracy, these standards must be monitored for degradation and quantitated accurately using a secondary method, typically spectrophotometry. Additionally, choosing a proper quantitation standard that amplifies similarly to the AAV vector is challenging (1).

The QIAcuity uses digital PCR (dPCR) that works by dividing the PCR reaction into thousands of fixed, solid partitions in a QIAcuity Nanoplate. The template becomes randomly distributed across all available partitions of the nanoplate. Fluorescence signals are then measured at the end-point. Positive partitions containing the amplification target are detected by measuring the fluorescence signal of sequence-specific DNA probes or intercalating dyes. Poisson statistics is applied to calculate the average amount of target DNA per valid, analyzable partition. This value is used to calculate the amount of target DNA in the starting sample without the need for quantitation standards. This technology has a greater tolerance for inhibitors and tighter %CVs than qPCR (2).

Nanoplates are available with 26,000 partitions for applications where the target is rare and/or diluted and 8500 partitions in a 96-well format for high-throughput use with a concentrated template. Since AAV is usually produced with titers at or above $1x10^{12}$ genome copies/ml, the 8.5K Nanoplate is a good fit for this application. With such abundant targets, the sample must be diluted to fall within a concentration range that does not saturate all partitions with positive signals – ▷

having some negative partitions is a requirement for concentration determination. In the QIAcuity analysis software, the random distribution of partitions with positive and negative signals is analyzed using Poisson distribution to generate λ, the estimated number of copies of the target molecules per positive partition. This value is converted into copies/µl in the final reaction volume, and a 95% confidence interval is calculated for each concentration. The 95% confidence interval is minimized when λ is in the range of 0.5 to 3 due to lower partitioning and sub-sampling error (3) (Table 1).

Using an 8.5K Nanoplate with a partition size of 0.34 nl and a λ of 0.5, as an example, the minimum target molecules/µl in the final reaction should ideally be ~1500 (0.5/0.34 x 1000). When preparing AAV samples of unknown concentration, serially dilute samples 1:10 until at least one or more of the dilutions is expected to fall within the optimal range.

This study tested a workflow for quantitation and qualification of AAV samples using a duplex assay on the QIAcuity dPCR instrument targeting both an insert (GFP) and the viral backbone (AAV2-ITR). AAV2-ITR is one of the most widely used elements in AAV constructs. Assaying multiple targets across the AAV virus can be advantageous since the differing values may be used as a quality indicator. Disproportionately high detection of one target over another may point to an issue in manufacturing. Assays up to 5-plex can be run on the QIAcuity instrument for quantitation when using fluorescent probes.

The two ITR regions form hairpin-like secondary structures, leading to accessibility issues for the primers/probe and causing a reduction in quantitation values. Using a restriction enzyme to cut within the secondary structure can improve amplification in these regions. In this study, the restriction enzyme, Msp I was used, which cuts the secondary structure in both ITRs and one or more times within the AAV genome resulting in two separate copies of the ITR. The decision to use a restriction enzyme is based on the specific samples and assay being used. A non-linearized plasmid should be cut before running on the QIAcuity. Although there are several restriction enzymes with high activity, tests showed that when added to the QIAcuity Probe Master Mix, the Msp I enzyme was not compatible, so digestion was performed before the dPCR reaction setup.

For AAV PCR assays that work well without restriction digest, it is not uncommon to add a 95°C, 10-minute capsid lysis step to the PCR cycling conditions to reduce

Table 1. 95% confidence intervals (CI) and λ for 8 dilutions of an AAV sample. Although the CI for the final dilution is approaching 50%, keep in mind that fewer copies are detected, so the range of copies within the 95% CI is still small at 5.2 to 8.4 copies/µl.

Sample	QIAcuity concentration (copies/µl)	Total copies in reaction	95% CI	Negative partitions	λ (copies/partition)	%CV (n=3 wells)
Dil 1	9678.0	116136.0	1.2%	4.5%	3.101	0.83%
Dil 2	7508.8	90105.6	1.4%	9.6%	2.343	1.91%
Dil 3	2290.6	27487.2	2.6%	49.7%	0.699	1.47%
Dil 4	669.6	8035.2	4.8%	81.5%	0.205	1.53%
Dil 5	200.0	2400.0	8.8%	94.2%	0.060	1.12%
Dil 6	67.7	812.4	15.1%	98.0%	0.020	3.76%
Dil 7	24.4	292.8	25.0%	99.2%	0.008	19.54%
Dil 8	6.8	81.6	47.4%	99.8%	0.002	12.37%

pre-treatment steps. During testing of the GFP assay, heated capsid lysis was performed on the QIAcuity and compared to heated lysis in advance of the reaction setup. A 50% decrease in AAV concentration was observed for the lysed samples on-board the QIAcuity indicating the capsid lysis was likely in complete. Capsid lysis is critical for accurate quantitation, so one must optimize the lysis step.

There are many ways to prepare AAV samples for dPCR quantitation. The assay workflow described in this application note can be used as a reference or starting point.

Materials and Methods

Four ssAAV control samples (Vector Biolabs and Vigene Biosciences) were used from three different serotypes with green fluorescent protein (GFP) inserts and ITR sites derived from the AAV-2 serotype to test the workflow. The samples were set up in a duplex assay targeting the ITR region and GFP insert using published qPCR assays with modifications to the fluorophore/quencher to allow multiplexing (4, 5). The AAV2 ITR assay uses a hydrolysis probe labeled with fluorescein (FAM) and quenched with BHQ-1, whereas the GFP assay uses a hydrolysis probe labeled with HEX and quenched with BHQ-1. The AAV2-ITR assay was designed to amplify both single-stranded AAV and double-stranded vector plasmids.

Samples were first treated with DNase (NEB, M0303) to remove any DNA not contained within the viral capsid (Table 2). Before setting up the DNase reaction, 10% Pluronic™ F-68 surfactant (Thermo Fisher, 24040-032) was diluted to 1% with water. Pluronic F-68 is a common additive used to improve stability and prevent titer loss due to AAV binding to plastic surfaces. The DNase reactions were incubated at 37°C for 30 minutes.

Table 2. DNase digest setup

Component	Volume per 50 µl reaction	Final concentration
10X DNase I reaction buffer (NEB)	5 µl	1X
1% Pluronic F-68	5 µl	0.1%
DNase I (2 U/µl)	5 µl	0.2 U/µl
AAV sample	5 µl	
Nuclease-free water	30 µl	

The samples were then serially diluted in a low Tris buffer containing poly-A carrier RNA (QIAGEN, 1017647) and Pluronic F-68 (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 ng/µl carrier RNA, 0.01% Pluronic F-68). Serially diluted samples were heated at 95°C for 10 minutes in a thermal cycler to break open the capsids. This step was followed by a 5-minute cool down to room temperature. After lysis, samples were digested with Anza™ Msp I restriction enzyme (Thermo Fisher, IVGN0656) at 37°C for 15 minutes (Table 3).

Table 3. Restriction digest

Component	Volume per 20 µl reaction	Final concentration
10X Anza Buffer	2 µl	1X
Anza 65 Msp I (20 U/µl)	0.25 µl	0.25 U/µl
DNase I-treated AAV sample	10 µl	
Nuclease-free water	7.75 µl	

Following the restriction digest, 5 µl of the digested AAV was setup in a 12 µl QIAcuity Probe PCR reaction (Table 4). ▷

Table 4. QIAcuity Probe PCR reaction setup

Component	Volume per 12 µl reaction	Final concentration
4x Probe PCR Master Mix	3 µl	1X
10x AAV2-ITR primer-probe mix	1.2 µl	0.8 µM forward primer 0.8 µM reverse primer 0.4 µM probe
10x GFP primer-probe mix	1.2 µl	0.8 µM forward primer 0.8 µM reverse primer 0.4 µM probe
RNase-free water	1.6 µl	
Digested AAV sample	5 µl	

The reactions were mixed and transferred to a QIAcuity Nanoplate 8.5K 96-well, sealed and loaded onto a QIAcuity Four instrument. Partitioning of the reactions, PCR cycling and imaging are performed automatically by the instrument. Cycling conditions consisted of a 95°C hot start for 2 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds. The nanoplate was imaged at a 500 ms exposure and gain 6 on both the green and yellow channels. Analysis was performed using the QIAcuity Software Suite v1.2. A reference dye is included in the master mix, allowing the software to determine which partitions are valid and analyzable. A volume precision factor (VPF) was applied to the quantitation data to account for variations in partition size for different nanoplate batches. More details on the VPF can be found in the *QIAcuity User Manual* at **www.qiagen.com**.

Results and Discussion

Obtaining reproducible, accurate and precise quantitation results that do not vary significantly from day to day or from user to user is critical for implementing a new assay.

Comparison to qPCR

All AAV samples were extracted and quantified by qPCR with the components from the AAVpro® Titration Kit Ver.2 (Takara Bio, 6233) to compare the detected titers to QIAcuity dPCR. This kit amplifies the AAV-2 derived ITR region with detection based on fluorescence of TB Green intercalating dye. The positive control, a plasmid containing two copies of the ITR, is serially diluted and used as a quantitation standard for the qPCR assay. The detected titer of the samples was approximately 30% of the expected titer given by the manufacturers. Several publications have mentioned encountering challenges with accurately quantitating AAV using a plasmid standard. Hence, a full capsid AAV-2 Reference Standard (Vigene, RS-AAV2-FL) was serially diluted after DNase treatment and capsid lysis with the AAVpro Titration Reagents and used as a quantitation standard. This brought the detected titers up to approximately 65% of expected values, but the standard curve values were not optimal (R^2 0.9997, slope –2.84, PCR efficiency 1.25) (Figure 1). Higher than expected slope and efficiency values can indicate PCR inhibition; in this case, it may be due to the secondary structure of the ITR sites. When the AAV-2 Reference Standard and the samples were cut with an Msp I enzyme, the titers increased to 71% of the expected titer with good standard curve values (R^2 0.9989, slope –3.28, PCR efficiency 1.01). The differing titer values and challenges with the standard illustrate one of the drawbacks of qPCR – accurate quantitation relies on a high-quality standard with identical amplification characteristics to the target samples. Any drop in standard concentration due to storage effects, improper pipetting, etc., will cause a corresponding change in the detected titer of the unknowns.

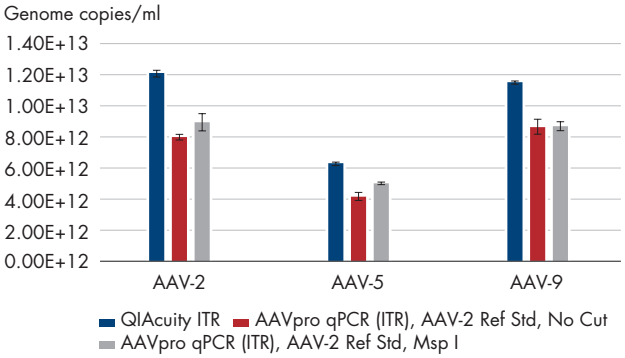

Figure 1. Detected titers (genome copies/ml) with QIAcuity dPCR and with the AAVpro ITR qPCR assay with and without Msp I digest.

Intra-assay and inter-assay reproducibility

The AAV samples were pre-treated, serially diluted and quantitated with the ITR/GFP duplex assay on the QIAcuity (Figures 2–3) over six days – the average intra-day %CV across all samples was 1.7 (Table 5). During testing, 3 serial dilutions were assayed in triplicate and used to calculate the genome copies/µl of the stock sample. The intra-day %CV across all dilutions, ranging from approximately 1000 copies/µl to 8000 copies/µl in the final reaction mix, was 2.6 for the GFP assay and 3.5 for the ITR assay. The QIAgility®, an automated liquid handler, performed all pipetting steps, including serial dilutions in a follow-up test. When using automation, the intra-run CVs across dilutions was 1.0 for the GFP assay and 1.7 for the ITR assay.

Table 5. The intra-assay reproducibility from six independent assays

	ITR assay		GFP assay	
Sample	% CV	CV range	% CV	CV range
AAV-2, 1	1.7	0.6–3.3	1.9	0.9–2.7
AAV-2, 2	1.8	1.2–2.4	1.7	0.4–2.3
AAV-5	1.8	1.1–2.7	1.7	0.8–2.6
AAV-9	0.9	0.4–1.8	1.8	1.0–2.9

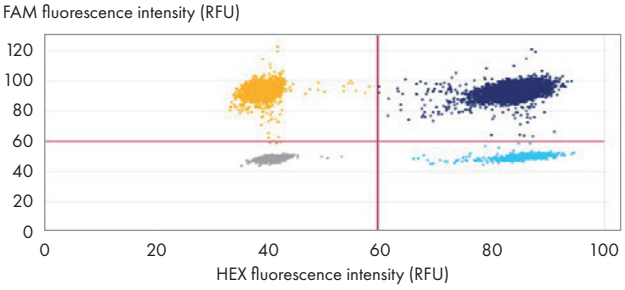

Figure 2. 2D plot of detected fluorescence for the GFP assay (X-axis) versus the ITR assay (Y-axis) for an AAV-2 sample. Positive and negative partitions can be separated easily with a threshold, and assays are clean with minimal rain.

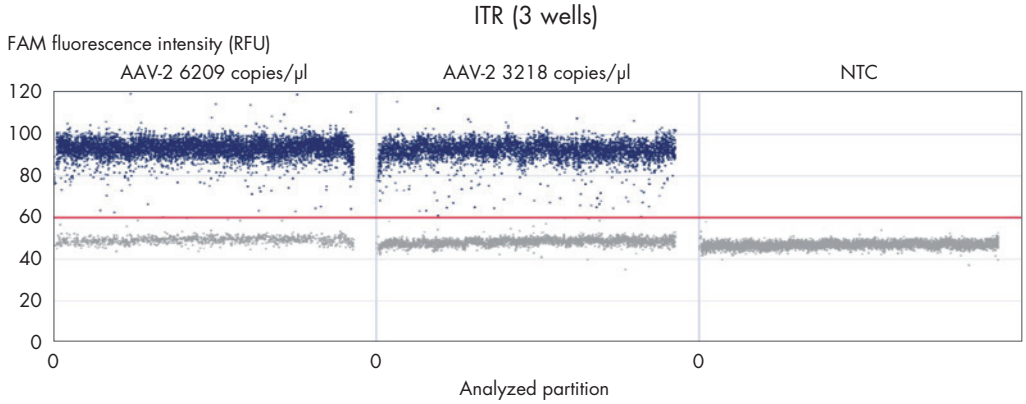

Figure 3. Example 1D plot showing positive and negative partitions for a diluted AAV-2 sample with the ITR dPCR assay in the first two wells and an NTC in the last well.

The nominal titers of the samples were 1.00E+13 genome copies/ml as specified by the vendors' in-house qPCR assays. The titers, as detected by the QIAcuity, were 63 to 121% of expected. The inter-assay CV for the 4 samples ranged from 2.6 to 6.5 (Table 6). When quantified by qPCR, the same samples were also detected at a lower titer, but the ratio of dPCR:qPCR concentration was approximately 1.3X across all 4 samples, showing that both technologies were consistent in detection. If follow-up qPCR runs continue to show a ratio of 1.3X dPCR:qPCR, this number can be used as a conversion factor to convert existing qPCR quantitation data to dPCR.

To keep intra and inter-run %CVs low, it is important to use good pipetting techniques and well-calibrated pipettors. When performing serial dilutions, avoid using small template volumes that are difficult to pipette consistently. The use of additives like poly-A carrier RNA and Pluronic F-68 and low bind plastics and tubes can help reduce the loss of titer.

ITR/GFP ratio for qualification

Dividing the detected copies per µl of the ITR assay by the detected copies per µl of the GFP assay should result in a ratio of 2 when the ITR sites are separated by restriction digest. A pAAV-GFP plasmid generated a ratio of 2.04 ± 0.10 when quantified by dPCR. Figure 4 shows the ITR/GFP ratios for both AAV digested with an Msp I and AAV left uncut. The AAV2-1 sample consistently generated a high ratio of 2.70 ± 0.09, which may warrant closer inspection to identify any manufacturing issues. The uncut AAV has a ratio of close to 1.0, which is expected since the fully intact ITR sites are connected and would remain in the same partition.

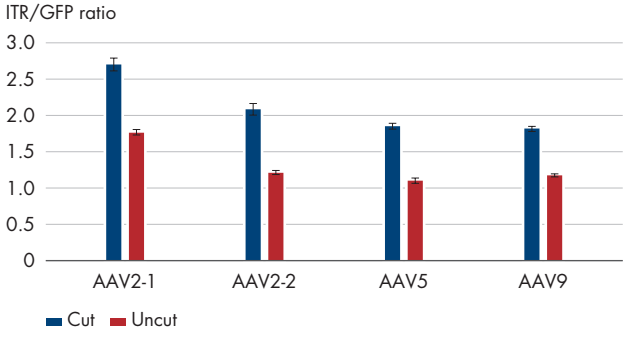

Figure 4. ITR/GFP ratio for AAV samples with and without Msp I digest.

Table 6. Inter-assay reproducibility (n=7) over six days using two different technicians. On the seventh day of setup, the QIAgility, automated liquid handler was used instead of manual pipetting. The detected titer from qPCR is shown as a comparison – the QIAcuity titer is approximately 1.3X higher than the qPCR titer.

Sample	GC/ml QIAcuity dPCR (ITR)	QIAcuity inter-assay %CV	GC/ml AAVpro ITR qPCR	Ratio dPCR:qPCR
AAV-2, 1	1.21E+13	5.3	8.95E+12	1.35
AAV-2, 2	7.57E+12	2.6	5.89E+12	1.29
AAV-5	6.26E+12	3.9	5.00E+12	1.25
AAV-9	1.15E+13	6.5	8.69E+12	1.33

Conclusion

With very low intra-assay and inter-assay CVs <6.5%, we demonstrate one of the main benefits of dPCR: reproducibility. A duplex dPCR probe assay that amplifies both a backbone target and a gene of interest (GOI) provides additional information about AAV, which may aid in the qualification of samples. Some QIAcuity instruments can detect fluorescent signals in 5 different channels allowing even more multiplexing, which may be valuable in further qualifying AAV genome integrity. Existing, well-characterized qPCR assays can typically be transferred to dPCR on the QIAcuity instrument with very little optimization.

References

1. 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**(2),115-125.
2. Dingle, T.C. et al. (2013) Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances. Clinical chemistry. **59**(11), 1670–1672.
3. Basu, A.S. (2017) Digital assays part I: partitioning statistics and digital PCR. SLAS TECHNOLOGY: Translating life sciences innovation. **22**(4), 369–386.
4. Aurnhammer, C. et al. (2012) Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Human gene therapy methods. **23**,18–28.
5. Delenda, C. and Gaillard, C. (2005) Real-time quantitative PCR for the design of lentiviral vector analytical assays. Gene ther. **12**, S36–S50.

Ordering Information

Product	Contents	Cat. no.
QIAcuity Probe PCR Kit (5 ml)	5 x 1 ml 4x concentrated QIAcuity Probe Mastermix, 8 x 1.9 ml Water	250102
QIAcuity Nanoplate 8.5K 96-well (10)	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
QIAgility System HEPA/UV (incl. PC)	Instrument and service agreement package: robotic workstation for automated PCR setup (with UV light and HEPA filter), notebook computer and QIAgility software; includes installation, application training and one-year warranty on labor, travel and parts	9001532
QIAcuity Four Platform System	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit, 1 year warranty on labor, travel, and parts	911042

The products mentioned here are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or user operator manual. QIAGEN instructions for use and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services (or your local distributor).

For more information, visit: **www.qiagen.com/dPCR**