Application Note

Optimized in-process recombinant adeno-associated virus (rAAV) vector genome titer protocol using the QIAcuity® Digital PCR System

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Protocol was developed, optimized, and evaluated for use with the QIAcuity® dPCR system by National Resilience, Inc.

Introduction

Recombinant adeno-associated virus (rAAV) vectors are widely used for in vivo gene therapies (1). These complex drug products represent potentially life-saving treatments for millions of people; but to realize this potential, rAAV vector manufacturing must reach scales far exceeding what's possible today. This creates an urgent need for new processes that improve the purity, potency, and yield of rAAV vectors.

A precise vector genome titer quantification is a particularly crucial step in viral vector production, as clinical dosing of rAAV depends on vector genome concentration. Digital PCR provides superior accuracy to qPCR without the need of a standard curve; however, Droplet Digital PCR (ddPCR) workflows are laborious and thus limited by throughput. Here we provide an integrated rAAV genome titer method using the QIAcuity Digital PCR (dPCR) System with detailed parameters for high assay performance.

Using this optimized method for pre-PCR handling of in-process rAAV samples, the results demonstrated that QIAcuity dPCR system generates the same level of accuracy and precision as the current gold standard ddPCR system but with much faster sample-to-result times (2 hours vs 7 hours) and higher overall throughput and scalability.

Instruments and Materials

Instruments

Instrument	Manufacturer	Cat. No.
QIAcuity Eight dPCR system	QIAGEN	911052
QXOne Droplet Digital PCR System	Bio-Rad	12006536

Materials

Material	Manufacturer	Cat. No.
Molecular Grade Water	various	10972-015
1x TE Buffer	various	various
10X PCR Buffer and 25mM $MgCl_2$	Applied Biosystems	4379878
10% Pluronic F-68	Gibco	24040-032
Purelink PCR Purification Kit	Invitrogen	K310001
PCR Purification Kit	various	various
DNaseI and 10x Buffer	NEB	M0303S
Qubit 1x dsDNA High Sensitivity (HS) kit	Invitrogen	Q33230
QIAcuity Nanoplates, 8.5k 96-well	QIAGEN	250011
QIAcuity Nanoplates, 26k 24-well	QIAGEN	250002
qPCR Proteinase K Buffer	Teknova	2P0355
500 mM EDTA	Invitrogen	15575020
QIAcuity Probe PCR Kit	QIAGEN	250103
Custom 20X Primers/Probe mix (gene of interest*)	IDT/custom	N/A
96 well PCR plate	various	various
pAAV-ZsGreen1 Vector	Takara	6231
In-process AAV2-ZsGreen1 intermediate	Resilience	N/A

* Targeting ZsGreen1 in this study

Methods

Reagent preparation:

1. Prepare fresh 1X pre-dilution buffer for each assay (Table 1). Volume may be scaled as necessary.

Table 1. 1X pre-dilution buffer set-up

Reagent	Vendor	Final concentration	Volume for 1 ml solution
10x PCR Buffer	Applied BioSystems	1X	100 µl
25 mM $MgCl_2$		1.5 mM	60 µl
10% Pluronic F-68	Gibco	0.05%	5 µl
Nuclease-free water	Multiple	N/A	835 µl
Total volume		N/A	1000 µl

2. Vortex.

Linearized plasmid preparation:

3. Digest 1 µg of purified pAAV-ZsGreen1 (Takara) plasmid with ScaI-HF (NEB) according to manufacturer's recommendation.
4. Purify ScaI-HF digested plasmid by PCR purification using a PCR purification kit following the manufacturer's recommendations.
5. Quantify the purified, linearized plasmid by Qubit and use Nanodrop to assess DNA purity. The concentration is further converted into copy number by:

$$\text{Number of copies} = \frac{(\text{amount of plasmid DNA in ng/ml}) * 6.0221\text{E}23 \text{ molecules/mole}}{\text{plasmid length in base pair} * 1\text{E}9 \text{ ng/g} * 650\text{g/mole})}$$

6. Dilute linearized plasmid to 1E10 copies/ml in 1X TE buffer.

rAAV sample preparation:

7. Prepare DNaseI Master Mix according to the number of rAAV samples to be tested (Table 2).

Table 2. DNase Master Mix set-up for single reaction

Reagent	per reaction (µl)
10x DNase Buffer	10
DNase I (2000 U/ml)	1
10% Pluronic F-68	0.5
Nuclease-free water	78.5
Diluted rAAV sample*	10
Total volume	100

* **Note:** For rAAV samples with expected titer higher than 1E10 copies/ml, we recommend diluting in 1X pre-dilution buffer prior to DNaseI digestion.

8. Incubate at 37°C for 30 minutes, proceed to capsid lysis step.
9. Prepare capsid lysis buffer (Table 3).

Table 3. Capsid lysis buffer set-up for single reaction

Reagent	per reaction (µl)
Teknova qPCR Proteinase K Buffer	48
500 mM EDTA	2
Total volume	50

10. Combine 50 µl of DNAse digested rAAV sample with 50 µl of Capsid lysis buffer for a 1:1 ratio.
11. Incubate capsid lysis reaction at 95°C for 10 minutes and then allow to cool down to 4°C.
12. Perform 4-fold (or 5-fold) serial dilution: Transfer 25 µl (or 20 µl) of the capsid lysed rAAV samples into 75 µl (or 80 µl) of 1X pre-dilution buffer, mix well. Repeat serially 3 more times down to 128x (or 125x).
13. Prepare dPCR Master Mix (Table 4). Scale as required to accommodate the number of samples and dilutions being analyzed. Include an additional 1-2 'safety' reactions to accommodate pipetting variation.

Table 4. dPCR Master Mix set-up for single reaction

Reagent	per reaction (µl)
4x QIAcuity Probe PCR Master Mix	6.25
20x Custom Primer Probe Mix	1.25
Diluted rAAV sample	n/a
Nuclease-free water	12.25
Total volume	20

14. Transfer 20 µl of dPCR Master Mix to appropriate number of wells in a sterile, 96-well PCR plate, next transfer 5 µl of serial diluted samples to each well containing dPCR QIAcuity Master Mix. Mix well by gentle pipetting.
15. Transfer 20 µl of final dPCR mixture into an 8.5k Nanoplate and proceed with the QIAcuity cycling conditions in Table 5. Approximate run time is 2 hours.

Table 5. dPCR reaction conditions and imaging settings

Cycling profile		
1x	95°C	2 min 0 s
40x	95°C	15 s
	60°C	30 s
End		

Cycling profile		
Channel	Exposure duration	Gain
Appropriate Channel	500 ms	6

Results and Discussion

High precision and accuracy measurements of purified plasmid on ddPCR and dPCR systems

To assess the accuracy and precision of the dPCR and ddPCR technologies, we performed a 2-fold serial dilution of the linearized pAAV-ZsGreen1 plasmid starting with a 1 x 10^{10} copies/ml stock. The resulting dilutions were analyzed on the QIAcuity (dPCR) Digital PCR System and the Bio-Rad QXOne ddPCR system (Figure 1).

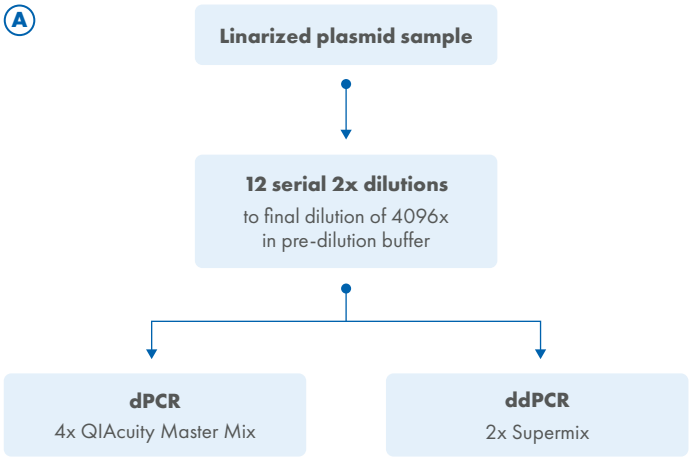
For both platforms, the resulting 1D and 2D scatterplots demonstrated adequate signal-to-noise ratios with negative and positive partition/droplet clusters clearly separated from one another (Figure 1 B). The QIAcuity dPCR system and Bio-Rad QXOne ddPCR system demonstrated highly similar performance in terms of precision and accuracy (Figure 1 B).

The ddPCR and dPCR systems deviated slightly in their sensitivity. Briefly, the lower limits of quantification (LLOQ) were assessed for each technology by determining ▶

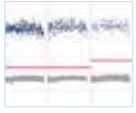
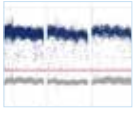
the lowest concentration capable of generating positive droplet (ddPCR) or partition (dPCR) clusters (Figure 1 B, C). The 26k nanoplate on the QIAcuity had the most sensitive LLOQ and the broadest linear dynamic range, followed by the QXOne, and then the 8.5k nanoplate on the QIAcuity (Figure 1 B, C). The highest concentration tested in this comparison study was 5000 copies/µl since this is the reported upper limit of quantification for ddPCR. Follow-up experiments are needed to define the true upper limit (ULOQ) of dPCR for this assay. Higher ULOQ are expected for dPCR given the previously reported 14,000 copies/µl ULOQ for the 8.5k nanoplate configuration partition format (2).

In summary, the 26k nanoplate, which contains 26,000 partitions per well, produced the widest linear range with LLOQ down to 2.5 copies/µl. This is of special importance in the quantification of low abundant targets such as contaminating sequences or low abundance variants. Additionally, the two platforms deviated significantly in sample-to-result time in this analysis (Figure 1 B).

A



B

	QXOne	QIAcuity 8.5k nanoplate	QIAcuity 26k nanoplate
Signal-to-noise			
Linear range (cp/µl)*	5 to 5,000	40 to >5,000	2.5 to >5,000
Precision	14%	10%	13%
Accuracy	108%	107%	108%
LLOQ (cp/µl)	5	40	2.5
Sample-to-result	7 hours	2 hours	2 hours

* Higher concentrations of target were not tested in this experiment.

C

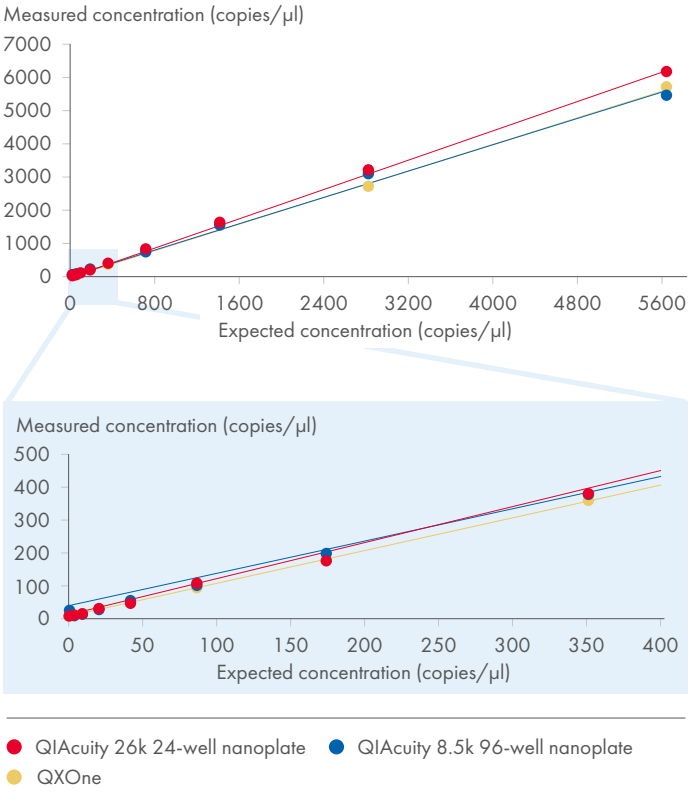


Figure 1. QIAcuity dPCR demonstrates improved performance to gold-standard ddPCR method in fraction of the time.
A Method Schematic. **B** Performance comparison between QIAcuity dPCR and Bio-Rad ddPCR.

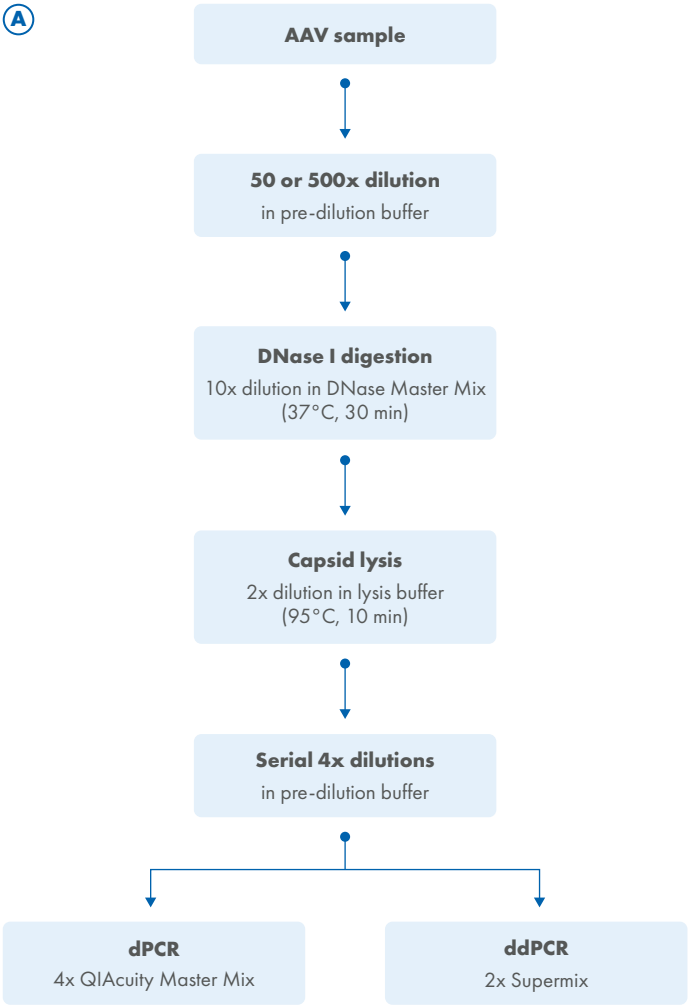
Comparable rAAV genome titer on dPCR and ddPCR systems

Comparable precision and accuracy of DNA quantification was demonstrated with purified, linearized plasmid DNA using dPCR (8.5k and 26k-format nanoplates) and ddPCR. Briefly, rAAV samples were first pre-diluted in PCR buffer then treated with DNaseI to degrade free, non-encapsidated DNA. Encapsidated rAAV DNA was then further released from the rAAV capsid by heating in the presence of salt and Sarkosyl (Figure 2 A).

Using the same primer and probe sets we quantified in-process rAAV samples on both instruments. Both dPCR and ddPCR utilize Poisson statistics to accurately quantify target molecules. Partition count is a key assay consideration when choosing a plate format as it directly impacts the statistical certainty of the data. For dPCR we selected the high throughput 8.5k 96-well nanoplate as it provides a sufficient number of integrated partitions per reaction for abundant targets (2). Since rAAV samples are highly concentrated and require pre-dilution to bring into analytical range, the 8.5K plate is the preferred choice for balancing performance and speed.

For the 7 samples analyzed on both platforms, we observed an average percent difference between dPCR and ddPCR of 13% and a difference in sample-to-result time of 5 hours (Figure 2 B). With similar performance to ddPCR, the QIAcuity's 2-hour sample-to-result time makes it better suited when a higher throughput set-up is desired (Figure 2).

A



B

In-Process AAV	dPCR		ddPCR		d vs. dd
Sample	Titer (vg/ml)	%CV*	Titer (vg/ml)	%CV	% Diff.
PC	4.65×10^{10}	17.3	5.27×10^{10}	10.3	12
S1	1.96×10^{10}	14.8	1.92×10^{10}	5.8	2
S2	2.24×10^{10}	14.7	2.12×10^{10}	2.7	6
S3	1.93×10^{10}	9.0	2.37×10^{10}	2.7	19
S4	1.76×10^{10}	8.5	2.30×10^{10}	5.6	23
S5	1.41×10^{10}	11.7	1.61×10^{10}	11.6	12
S6	1.40×10^{10}	9.5	1.71×10^{10}	13.2	18
(d)dPCR analysis time	2 hours		7 hours		5 hours

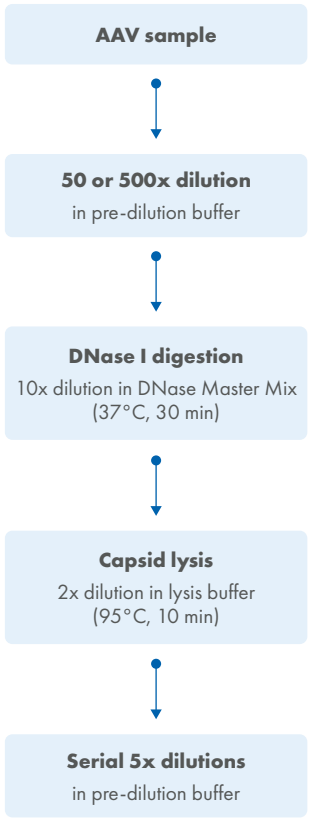
Figure 2. QIAcuity dPCR generates similar results to gold-standard ddPCR method in fraction of the time.
A Method Schematic. **B** QIAcuity dPCR and Bio-Rad ddPCR measured concentrations, %CV, and percent difference for the two methods for 6 linearized plasmid samples.

* The %CV was calculated from 4 single datapoints at 4-fold serial dilutions (4x 16x, 64x, and 128x after the capsid lysis step). Samples diluted to 128x post-capside lysis sometimes fell below the LLOQ of the 96-well, 8.5k dPCR nanoplates.

Optimized rAAV titer on QIAcuity

Initial comparisons of dPCR to ddPCR demonstrated similar performance and faster sample-to-result times for dPCR, but utilized a dilution scheme that was optimized for ddPCR. To further increase precision on the QIAcuity using the higher throughput 8.5k 96-well nanoplate, we optimized the dilutions to more appropriately fit within the range of this nanoplate. We tested 17 in-process rAAV samples ranging from harvest down through the final polished step, and found that by simply analyzing the rAAV samples at 25x and 125x in duplicate, all 4 measurements landed within the plate's range and resulted in CVs < 10%. Samples diluted 25x contained ~5000 vg/µl demonstrating accurate quantification in the upper range of the 8.5k 96-well nanoplate.

A



B

In-process AAV	Result (vg/ml)	%CV
S1	1.03×10^{11}	3.2
S2	1.04×10^{11}	8.7
S3	8.25×10^{10}	8.9
S4	1.85×10^{11}	3.2
S5	9.26×10^{10}	5.3
S6	4.11×10^{12}	4.8
S7	2.02×10^{10}	8.5
S8	1.27×10^{10}	4.0
S9	1.44×10^{10}	0.2
S10	3.49×10^{12}	3.0
S11	4.42×10^{12}	3.1
S12	4.27×10^{12}	3.6
S13	1.23×10^{11}	6.5
S14	1.41×10^{9}	1.5
S15	1.49×10^{9}	1.5
S16	4.50×10^{11}	0.5
S17	2.48×10^{11}	6.5

Figure 3. Reproducible rAAV quantification by QIAcuity dPCR.
A Method Schematic. **B** QIAcuity dPCR measured concentrations and %CV for 17 samples collected throughout the rAAV manufacturing process.

Conclusion

The QIAcuity dPCR system should play a major role in the development of new manufacturing processes by keeping pace with the analytical demands of iterative process refinements. In this study, we demonstrated comparable performance for an AAV titer assay run on both the QIAcuity dPCR system and the current gold-standard ddPCR system. Despite similar performance on the two platforms, 96 wells were processed in just 2 hours on the QIAcuity dPCR system compared to roughly 7 hours required for the same number of ddPCR wells.

Using a single QIAcuity Eight it's possible to load 16 plates in an 8-hour shift for a total of 1,536 wells. We look forward to exploring integration with high throughput liquid handlers and experimental multi-condition bioreactors to shorten time-to-data for rAAV process development.

Notes

References

1. Yang Lu, Recombinant adeno-associated virus as delivery vector for gene therapy--a review, Stem Cells Dev. 2004.
2. QIAcuity User Manual Extension: QIAcuity Application Guide.

Ordering Information

Product	Contents	Cat. no.
QIAcuity Probe PCR Kit	1 ml 4x concentrated QIAcuity Probe Master Mix, 2 x 1.9 ml Water	250101
QIAcuity Nanoplate 26k 24 well nanoplate	10 QIAcuity Nanoplates 26k 24-well, 11 Nanoplate Seals	250001
QIAcuity Nanoplate 8.5k 96 well nanoplate	10 QIAcuity Nanoplates 8.5k 96-well, 11 Nanoplate Seals	250021
QIAcuity Eight Platform System	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, nanoplate roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts	911052

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