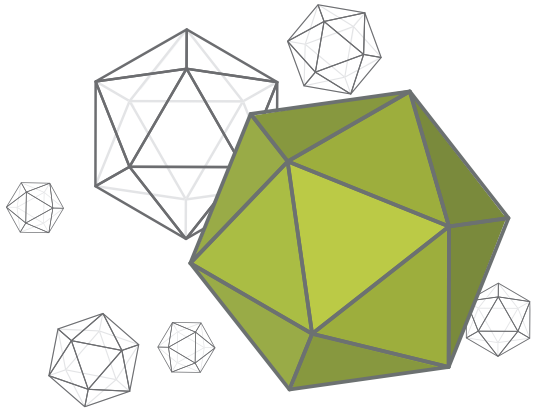
Viral vector quantification

Improved dPCR quantification of rAAV products using a salt active nuclease

Accurate quantification of recombinant adeno-associated viral vectors requires the complete degradation of non-encapsidated DNA



Introduction

Historically, titering of recombinant adeno-associated viral (rAAV) vector products has suffered from inconsistent results. A major cause of this variability is the incomplete digestion of non-encapsidated DNA due to rAAV particle aggregation. Complete disaggregation can only be achieved under conditions that inhibit the activity of most nucleases. However, utilizing a salt active nuclease (SAN) in a high-salt solution promotes both disaggregation and the complete digestion of non-encapsidated DNA in rAAV preparations.

In this technical note, we demonstrate the use of SAN treatment prior to measuring encapsidated rAAV genomes on the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System. This workflow employs crude lysates, requiring no sample extraction prior to quantification, thereby reducing hands-on time and permitting batch characterization before proceeding with purification.

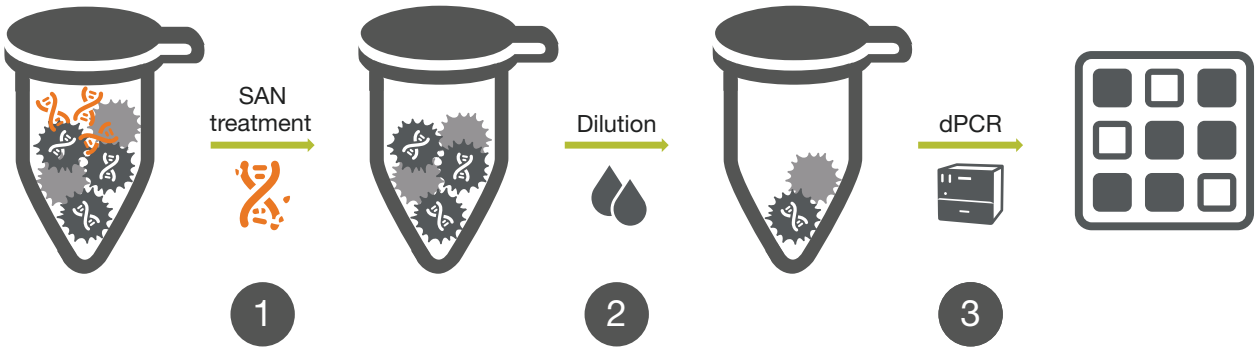


Figure 1. SAN digestion workflow. First, rAAV crude lysates are subjected to SAN treatment under high-salt conditions, which favor disaggregation. Next, the sample is diluted, then encapsidated genomes are measured by dPCR on the QuantStudio Absolute Q dPCR system using an assay (primer and probe set) that targets the transgene in the sample.

Methods

Reagents

The formulations for Salt Dilution Buffer, SAN Nuclease Buffer (2X), SAN Inactivation Solution, and dPCR Dilution Buffer are provided in Tables 8–11.

Crude lysate preparation

Gibco™ AAV-MAX Lysis Buffer was added to the culture flask at a 1:10 dilution (3.3 mL of AAV-MAX lysis buffer to 30 mL of culture), then the flask was swirled to mix[1]. Next, the flask was incubated at 37°C for 2 hours on an orbital shaker. The lysate was clarified by centrifugation at 4,500 × *g* for 30 minutes at 4°C (small volumes can be centrifuged at 13,000 × *g* for 10 minutes at 4°C). After centrifugation, the supernatant containing the crude lysate was transferred to a new tube[2].

Crude lysate dilution

Crude lysates were diluted in Salt Dilution Buffer (Table 8) according to their predicted titers, then kept on ice. The dilution scheme used is provided in Table 1.

SAN digestion

SAN Reaction Mix was prepared according to Table 2. For each sample, 10 µL of diluted crude lysate was combined with 90 µL of SAN Reaction Mix in a 96-well plate.

Table 2. SAN Reaction Mix

Component	Volume per reaction	Volume for 10 reactions*
Nuclease-free water	39 µL	429 µL
2X SAN Nuclease Buffer (Table 9)	50 µL	550 µL
Heat-Labile Salt Active Nuclease (25 U/µL)	1.0 µL	11 µL
Sample	10 µL	–
Total Volume	**100 µL**	**990 µL**

*Volumes may be scaled as needed depending on the number of samples to be treated. Volumes include 10% overage.

Each reaction was mixed by pipetting up and down without creating bubbles. The plate was sealed using Applied Biosystems™ MicroAmp™ Clear Adhesive Film, then centrifuged at 900 × *g* for 2 minutes to collect the liquid at the bottom of the well. Finally, the plate was incubated in a thermal cycler for 30 minutes at 37°C with the heated lid at 105°C, followed by a 4°C hold.

SAN inactivation

After incubation, the SAN Digestion plate was centrifuged at 2,000 × *g* for 30 seconds. Then, 100 µL of SAN Inactivation Solution (Table 10) was added to each well, followed by pipette mixing. The sealed plate was incubated at room temperature (15°C to 30°C) for 10 minutes.

Table 1. Dilution of crude lysates

Predicted titer*	Dilution factor	Intermediate dilution		Final dilution	
		Sample volume	Salt Dilution Buffer volume	Sample volume	Salt Dilution Buffer volume
1.00×10^{11} genome copies/mL	10	–	–	10 µL crude lysate	90 µL
5.00×10^{11} genome copies/mL	50	–	–	10 µL crude lysate	490 µL
1.00×10^{12} genome copies/mL	100	–	–	10 µL crude lysate	990 µL
5.00×10^{12} genome copies/mL	500	10 µL crude lysate	490 µL	10 µL Intermediate Dilution	90 µL
1.00×10^{13} genome copies/mL	1,000	10 µL crude lysate	990 µL	10 µL Intermediate Dilution	90 µL
5.00×10^{13} genome copies/mL	5,000	10 µL crude lysate	490 µL	10 µL Intermediate Dilution	990 µL
1.00×10^{14} genome copies/mL	10,000	10 µL crude lysate	990 µL	10 µL Intermediate Dilution	990 µL

*If the predicted titer fell between two concentrations, the higher dilution factor (i.e., more dilute sample) was used.

[1]If proceeding to a downstream workflow, $MgCl_2$ to a final concentration of 2 mM and Thermo Scientific™ Pierce™ Universal Nuclease or Benzonase™ to a final concentration of 90 U/mL can be added to the flask.

[2]Crude lysates can be stored at 2°C to 8°C for up to 4 weeks until processing or at ≤–70°C for long-term storage.

Digital PCR

After SAN inactivation, the samples were diluted in dPCR Dilution Buffer (Table 11) according to Table 3, then mixed by vortexing.

Table 3. Sample dilution for dPCR

Dilution ID	Sample volume	dPCR Dilution Buffer volume
Dilution 1	10 µL SAN-inactivated sample	490 µL
Dilution 2	250 µL Dilution 1	250 µL
Dilution 3	250 µL Dilution 2	250 µL

Table 4. dPCR reaction mix

Component	Volume per reaction	Volume for 16 reactions*
Nuclease-free water	2.5 µL	44 µL
Absolute Q Universal DNA Digital PCR Master Mix (5X)	2 µL	35.2 µL
Assay (20X)**	0.5 µL	8.8 µL
Dilution 3 of sample (from Table 3)	5 µL	–
Total Volume	**10 µL**	**88 µL**

*Volumes include 10% overage.
**Adjust volume based on assay concentration.

dPCR was performed using the Applied Biosystems™ Absolute Q™ Universal DNA Digital PCR Master Mix according to Table 4. Assay DT2W7KW was used for enhanced green fluorescent protein (eGFP) gene detection, Assay DTH49PU was used for AAV inverted tandem repeat sequence (ITR2) detection, and custom assays were used for genes of interest (GOIs) and CD19 gene detection.

Results and conclusions

Characterized rAAV preparations were treated with SAN nuclease, and the concentrations were determined on the QuantStudio Absolute Q dPCR system. The concentrations determined using the method described here were compared with the values provided on the accompanying Certificate of Analysis (CoA). As shown in Figure 2, different rAAV products

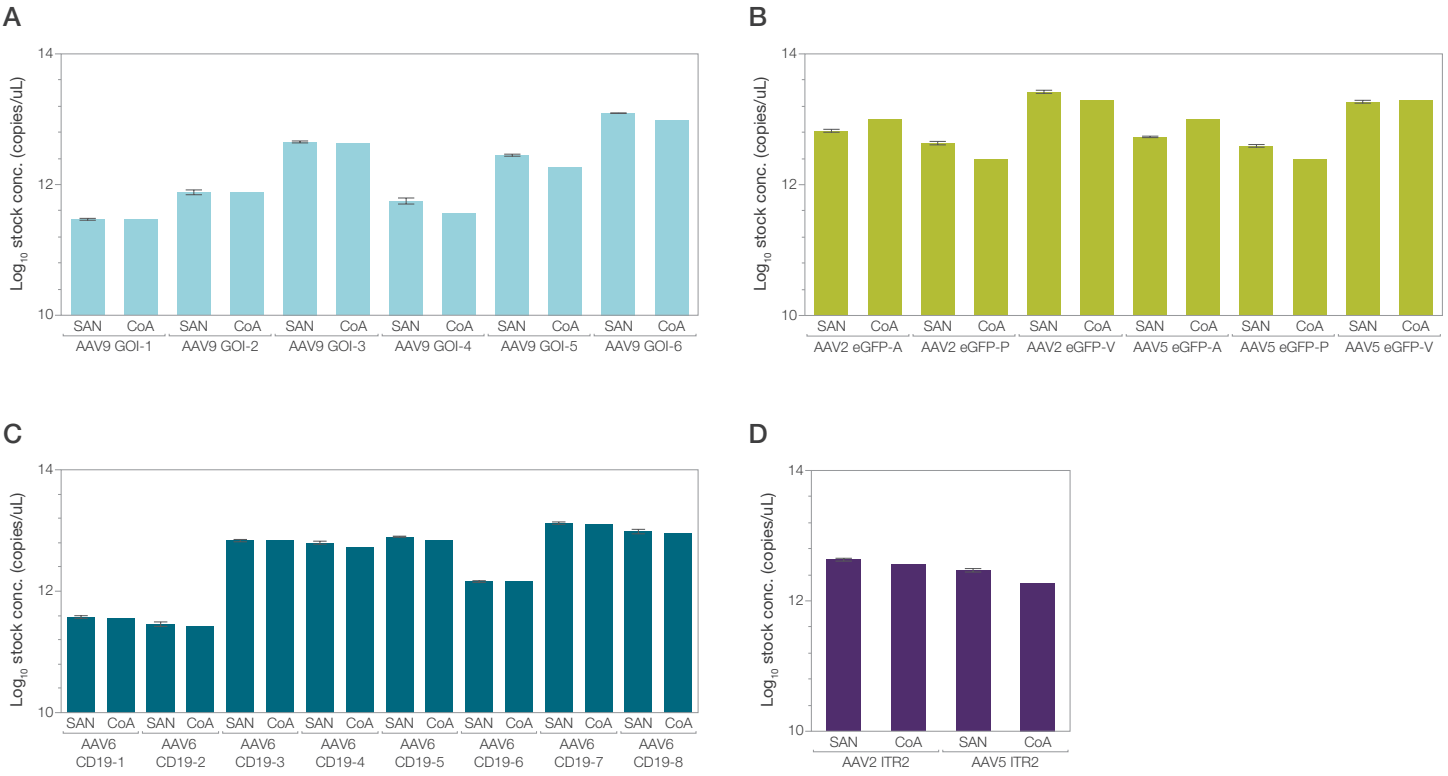


Figure 2. Performance of SAN workflow versus CoA. rAAV preparations were subjected to the SAN workflow described in this technical note and quantified on the QuantStudio Absolute Q dPCR system. Concentrations determined using the SAN workflow are represented as the mean of multiple replicates, and error bars indicate standard deviations. Values reported on the CoA are shown for comparison. **(A)** Detection of GOIs across six different AAV serotype 9 (AAV9) products. **(B)** Detection of eGFP across AAV serotypes 2 (AAV2) and 5 (AAV5) from three different vendors (A, P, and V). **(C)** Detection of CD19 across eight different AAV serotype 6 (AAV6) products. **(D)** Detection of ITR2 in AAV2 and AAV5.

from different sources and in different AAV serotypes showed good agreement with the concentrations provided on the CoA.

The quantification workflow described here does not require purification or sample extraction prior to dPCR, giving you the ability to gauge the success of an rAAV production run before undertaking extensive purification procedures.

Guidance and recommendations

dPCR reaction replicates

In addition to running multiple dilutions of each sample, we recommend running multiple dPCR replicates to help ensure accurate quantification.

Materials

The materials listed in Table 5 were used for testing. Substitutions, such as a different catalog number or manufacturer, use of strip tubes instead of PCR plates, or use of an incubator instead of a thermal cycler, may produce equivalent results but were not tested. Materials are available through **thermofisher.com** unless otherwise specified. General laboratory equipment and consumables are available from **fisherscientific.com** or another major laboratory supplier.

Controls

A list of recommended controls is provided in Table 6. In order to cross-compare dPCR results, control reactions should contain the same theoretical concentration of DNA, accounting for all dilution steps. It is highly recommended to quantify synthetic DNA controls using dPCR.

Additional resources

A list of additional information and technical resources is provided in Table 7.

Table 5. Ordering information

Description	Catalog number
Equipment	
Applied Biosystems QuantStudio Absolute Q Digital PCR System	A52864 A53267
Consumables	
Applied Biosystems QuantStudio Absolute Q MAP16 Plate Kit	A52865
Applied Biosystems MicroAmp Optical 96-Well Reaction Plate	N8010560 or equivalent
Applied Biosystems MicroAmp Clear Adhesive film	4306311
Reagents	
Applied Biosystems Absolute Q Universal DNA Digital PCR Master Mix (5X)	A72710
Heat-Labile Salt Active Nuclease (HL-SAN), 25 U/µL	**Fisher Scientific:** NC1709204 **ArcticZymes:** 70910-202
10X DNase I Buffer	**Fisher Scientific:** 50-995-020 **New England Biolabs:** B0303S
Invitrogen Tris (1 M), pH 8.0, RNase-free	AM9855G AM9856
Invitrogen EDTA (0.5 M), pH 8.0, RNase-free	AM9260G AM9261 AM9262
Invitrogen NaCl (5 M), RNase-free	AM9759 AM9760G
Gibco Poloxamer 188 Non-ionic Surfactant (10%)	24040032
Invitrogen 10 mg/mL Salmon Sperm DNA, Sheared	AM9680
Invitrogen UltraPure DNase/RNase-Free Distilled Water	10977015
Gibco AAV-MAX Lysis Buffer	A50520
Gibco AAV-MAX Helper-Free AAV Production System Kit	A51217
Thermo Scientific Pierce Universal Nuclease or Benzonase	88700, 88701, 88702, or equivalent
$MgCl_2$ solution, molecular biology grade	AM9530G or equivalent

Table 6. Recommended controls

Control	Purpose	Material	Expected result
AAV Positive Control	Demonstrates the accuracy and precision of the quantification method; results can be used to trend performance of quantification and preparation methods over time	Reference material prepared equivalently to sample	Stable quantification every time SAN digestion is performed
No Template Control (NTC)	dPCR contamination control; demonstrates no contamination during dPCR setup	Nuclease-free water added to dPCR reaction mix in place of SAN-digested sample	No amplification (or number of positive microreactions within method specifications)
DNA Positive Control	dPCR positive control; demonstrates dPCR performing as expected	DNA string or linearized plasmid corresponding to the amplicon sequence that is not subjected to SAN digestion. Either add DNA directly to the dPCR reaction or substitute Salt Dilution Buffer or SAN Nuclease Buffer (to a final concentration of 1X) for SAN Reaction Mix at the SAN Digestion step. Custom DNA strings and plasmids can be ordered from GeneArt Gene Synthesis Services.	Quantification within method specifications
SAN Control	Confirms complete SAN activity	DNA Positive Control that undergoes SAN Digestion	No amplification (or number of positive microreactions within method specifications)
SAN Inhibition Control	Confirms complete inactivation of SAN	DNA Positive Control to which SAN Inactivation Solution is added before adding SAN Reaction Mix	Quantification similar to DNA Positive Control (within method specifications)
SAN Negative Control	SAN contamination control; demonstrates no contamination during SAN treatment	Substitute Salt Dilution Buffer for sample during SAN Digestion step	No amplification (or number of positive microreactions within method specifications)

Table 7. Additional information and resources

Description	Publication number or link
Adeno-associated virus production resources	thermofisher.com/aav
Resources for using dPCR in cell and gene therapy development	thermofisher.com/us/en/home/life-science/pcr/digital-pcr/cell-gene-therapy-manufacturing
Invitrogen GeneArt Strings and custom plasmids	thermofisher.com/geneart
TaqMan pre-designed dPCR assays	thermofisher.com/taqman
Absolute Q digital PCR custom assay design tool	thermofisher.com/order/custom-assay-design-tool
Scientific poster: Fully integrated digital PCR system for robust and consistent viral titer quantification	Download poster
Scientific poster: Characterization of AAV Genomic Titer on the Applied Biosystems QuantStudio Absolute Q dPCR System	Download poster
Scientific poster: Measuring rAAV genomic titer, viral particle titer, and full/empty capsid ratio on the Applied Biosystems QuantStudio Absolute Q dPCR system	Download poster
Application note: Automated high-throughput digital PCR for accurate and consistent AAV quantification	Download application note
Technical note: Microfluidic array plate technology improves accuracy and consistency in digital PCR viral titer quantification for cell and gene therapy research	Download technical note
Flyer: Absolute Q Viral Titer Digital PCR Assays	Download flyer
Brochure: Gibco AAV-MAX Helper-Free AAV Production System	Download brochure

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Table 8. Salt Dilution Buffer

Component	Stock conc.	Volume	Final conc.
Nuclease-free water	N/A	44.215 mL	N/A
1M Tris, pH 8.0	1 M	500 µL	10 mM
0.5M EDTA, pH 8.0	0.5 M	10 µL	0.1 mM
5M NaCl	5 M	5 mL	0.5 M
10% Poloxamer 188	10% (w/v)	250 µL	0.05% (w/v)
Salmon Sperm DNA, sheared	10 mg/mL	25 µL	5 µg/mL
Total Volume		**50 mL**	

Table 9. SAN Nuclease Buffer (2X)

Component	Stock conc.	Volume	Final conc.
Nuclease-free water	N/A	2.975 mL	N/A
5M NaCl	5 M	1 mL	1 M
10X DNase I Buffer	10X	1 mL	2X
10% Poloxamer 188	10% (w/v)	25 µL	0.05% (w/v)
Total Volume		**5 mL**	

Table 10. SAN Inactivation Solution

Component	Stock conc.	Volume	Final conc.
Nuclease-free water	N/A	48.56 mL	N/A
1M Tris, pH 8.0	1 M	500 µL	10 mM
0.5M EDTA, pH 8.0	0.5 M	690 µL	6.9 mM
10% Poloxamer 188	10% (w/v)	250 µL	0.05% (w/v)
Total Volume		**50 mL**	

Table 11. dPCR Dilution Buffer

Component	Stock conc.	Volume	Final conc.
Nuclease-free water	N/A	49.215 mL	N/A
1M Tris, pH 8.0	1 M	500 µL	10 mM
0.5M EDTA, pH 8.0	0.5 M	10 µL	0.1 mM
10% Poloxamer 188	10% (w/v)	250 µL	0.05% (w/v)
Salmon Sperm DNA, sheared	10 mg/mL	25 µL	5 µg/mL
Total Volume		**50 mL**	

Learn more at **thermofisher.com/dpcr**