

AAV8 reference standard material.

Introduction

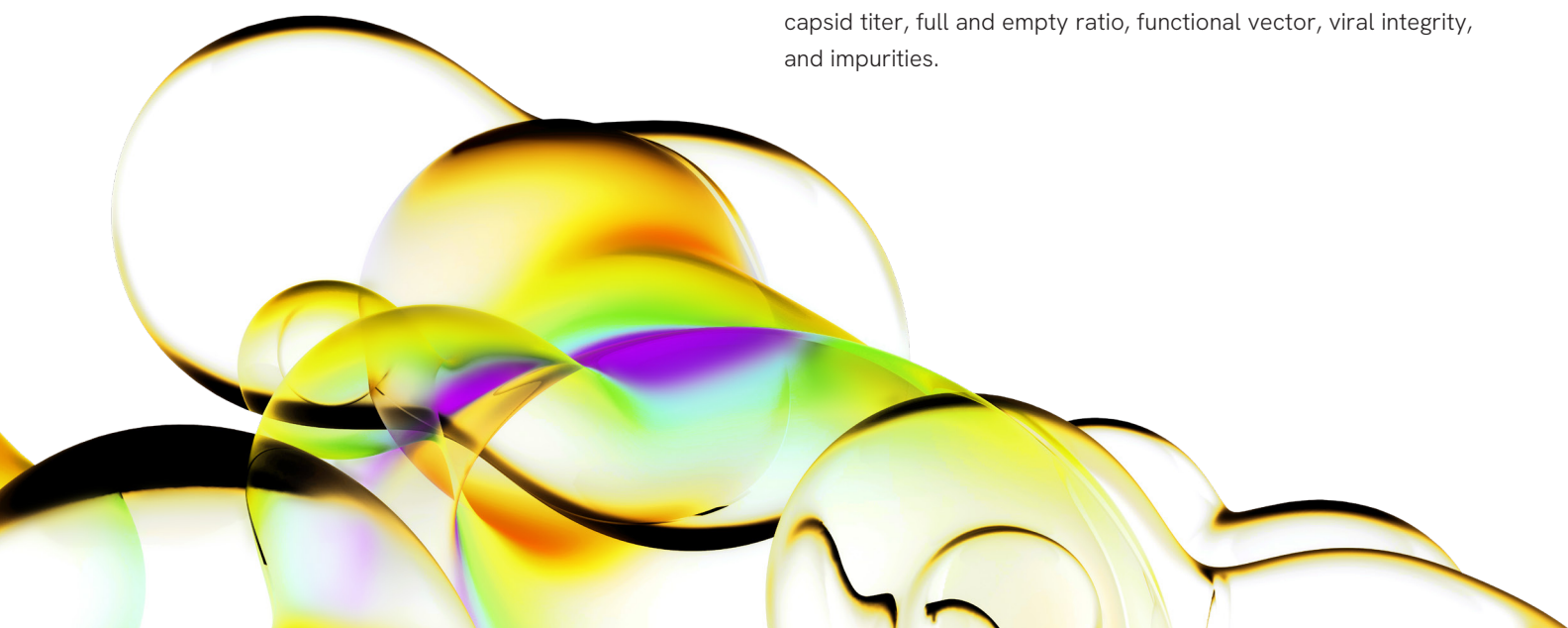
Reference materials are essential for standardizing assays. While there are multiple reference materials available, some provide only genomic, capsid and functional titer data, and others provide only full capsid percentages. The availability of the analytical information for these samples is also limited, which hinders comparisons between a laboratory's methodology and the reference material developer's methodology.

Revvity's AAV8 reference standard material (RSM) has been rigorously tested using a broad range of analytical assays with demonstrated reproducibility, which is complemented by detailed characterization data of the results for each method. This provides laboratories with reliable controls backed by comprehensive data for greater confidence in results.

Materials and methods

The material was produced in a suspension cell line cultured in a shaker incubator at 37 °C and 8% CO₂, transfected with a triple-plasmid system, containing a helper plasmid, a packaging plasmid (pAAV8 wild-type), and a transfer plasmid (pITR2-CMV-EGFP-WPRE). Three days after transfection, the material was lysed with a detergent-based buffer and an endonuclease for two hours. The material was clarified and then captured through affinity chromatography followed by iodixanol gradient ultracentrifugation, afterwards being formulated in DPBS and 0.001% poloxamer 188. After formulation, the material was diluted and aliquoted into 25 portions. This material was stored at -80 °C and thawed on the day of aliquoting into the final vials.

The assays tested fell into 6 different categories: genomic titer, capsid titer, full and empty ratio, functional vector, viral integrity, and impurities.



Most of the analyses were performed in only one location (unless otherwise stated). For assays of high variability and high interest, such as genomic titer measurement by qPCR, capsid titer by ELISA, and functional titer, multiple laboratories are participating in an interlaboratory evaluation of this material. The results from this study will be incorporated into this technical note upon completion.

Characterization results

Genomic titer

The average genomic titer was determined at $1.52\text{E}+12$ VG/mL $\pm 1.02\text{E}+11$ (SD), by ITR-targeting qPCR, dPCR, ddPCR, and EGFP-targeting ddPCR. The genome concentration was determined at a minimum in duplicate. For qPCR, 3 separate measurements in 2 different dilutions in duplicates were used for the titer measurement. For dPCR, 2 different dilutions in triplicates were used. For ddPCR, 2 different dilutions were used. More detailed information can be found in related application notes.

Table 1

	Assay	Measurement	Standard deviation
Genomic titer	Genomic titer ITR-qPCR (VG/mL)	1.50E+12	6.75E+10
	Genomic titer ITR-ddPCR (VG/mL)	1.49E+12	2.62E+09
	Genomic titer ITR-dPCR (VG/mL)	1.52E+12	3.35E+10
	Genomic titer EGFP-ddPCR (VG/mL)	1.58E+12	1.61E+10

Capsid titer

The average capsid titer was determined at $1.70\text{E}+12$ capsids/mL $\pm 5.27\text{E}+11$ (SD), by AlphaLISA™ AAV8 Detection Kit assay (Revvity, Part # AL3180HV), and AAV8 Titration ELISA (Progen Biotechnik GmbH, Article number PRAAV8). The capsid concentration by AlphaLISA assay was determined in 5 dilutions in triplicates, while determination through ELISA was performed at 2 dilutions in duplicates. More detailed information can be found in the manufacturer's manual.

Table 2

	Assay	Measurement	Standard deviation
Genomic titer	Capsid titer AlphaLISA™ assay (cap/mL)	1.78E+12	5.72E+11
	Capsid titer ELISA (cap/mL)	1.62E+12	3.34E+11

Full and empty capsid

The average full capsid was determined at $89.45\% \pm 6.10$ (SD), by comparison between qPCR and ELISA titers, spectrophotometry, cryo-transmission electron microscopy (cryo-TEM), and sedimentation velocity analytical ultracentrifugation (SV-AUC). Measurements by spectrophotometry, cryo-TEM, and SV-AUC were performed after 10-fold concentration in an ultrafiltration unit. The qPCR/ELISA full capsid was calculated by comparing all qPCR measurements to the average capsid titer and excluding values above 100%. Full capsid by spectrophotometry was performed in the Stunner instrument (Unchained Labs). Measurement was performed in triplicates. Full capsid by cryo-TEM was performed in a JEM-2200FS/CR TEM (JEOL) by CIC bioGUNE. SV-AUC measurement was conducted on an Optima Analytical Ultracentrifuge with an AN50Ti 8-hole rotor (Beckman Coulter) by Biofidus AG.

Table 3

	Assay	Measurement	Standard deviation
Full capsid	Percentage of full capsids qPCR/ELISA (%)	90.94%	7.08%
	Percentage of full capsids spectrophotometry (%)	88.18%	1.21%
	Percentage of full capsids cryo-TEM (%)	83.06%	5.33%
	Percentage of full capsids SV-AUC (%)	95.60%	N/A

Functional vector

The functional vector titer was determined at $3.58\text{E}+08$ TU/mL $\pm 3.91\text{E}+07$ (SD), through a fluorescence titration assay. Adherent HEK293 cells were seeded and transduced after one day in triplicates at $3.2\text{E}+04$, $6.4\text{E}+04$, and $1.28\text{E}+05$ viral genomes per cell at the time of transduction. Readings were performed 3 days post-transduction, using a Nucleocounter® NC-3000™ fluorescence image cytometer (Chemometec). The transduction titer was calculated using the following formula:

$$\text{Transducing units titer (TU/mL)} = \frac{N \times F \times D}{V}$$

- N = Total cells in the well at time of transduction
- F = Percentage of GFP+/PI- cells
- D = Dilution factor of the virus
- V = Virus volume in mL

Representative gating schemes are shown in figure 1.

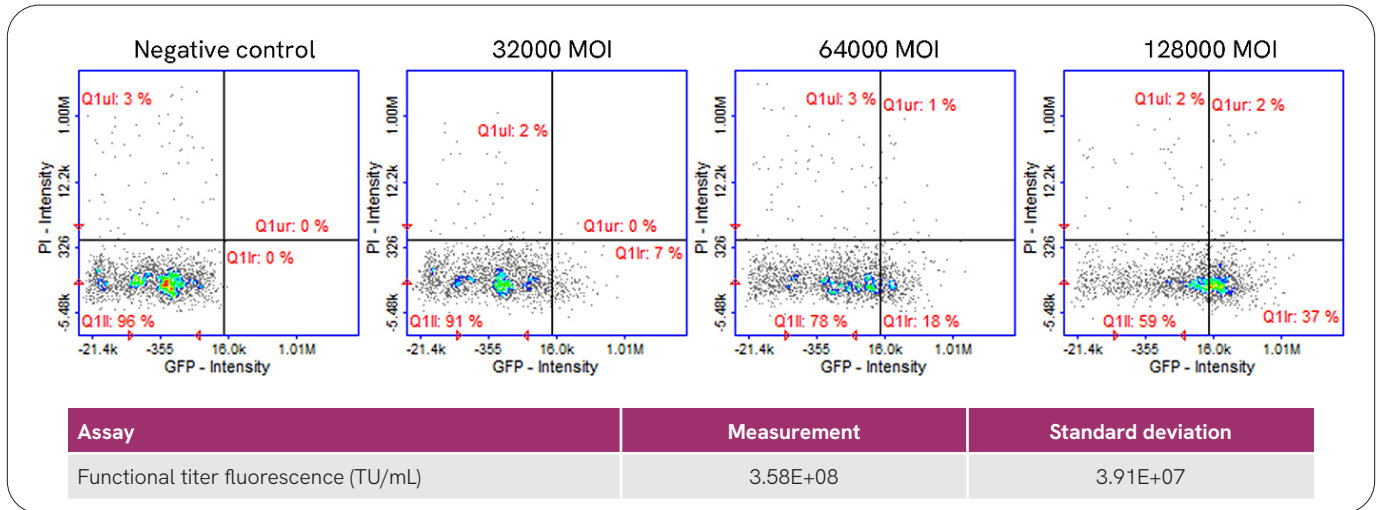


Figure 1

Viral integrity

Integrity was determined on the capsid and genomic level. An SDS-PAGE stained with silver showed the viral proteins (VP) 1, 2 and 3 as the only source of proteins in the sample, and all were intact at the expected molecular weights. An alkaline denaturing agarose gel showed the viral genome at the expected 3.3 knt, with no degradation or self-complementarity. The self-complementary AAV control can be seen at the expected near-double plasmid ITR-to-ITR length. Both gel images can be seen in figure 2.

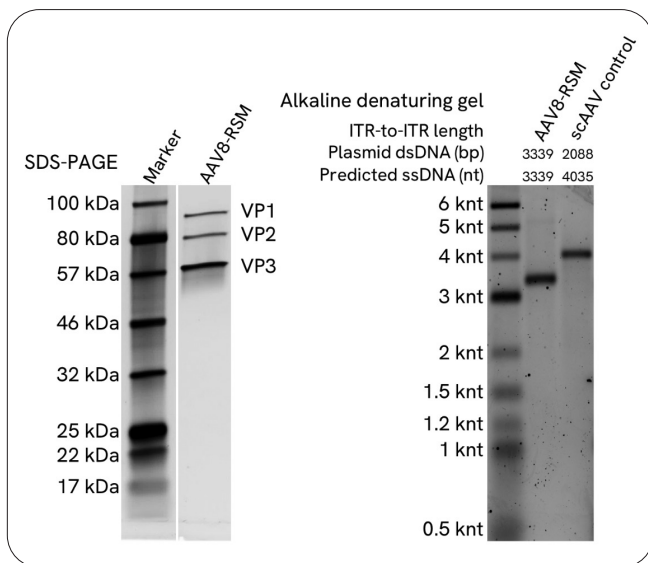


Figure 2

Impurities

Impurity analysis was determined at the values shown in table 4. The host-cell DNA was measured using the HostDetect™ HEK293 PCR DNA Quant Kit assay (Revvity) in 2 separate sites, in 2 different dilutions in duplicates. Next-generation sequencing (NGS) was performed through a long-read sequencing system (Oxford Nanopore Technologies). Plasmid DNA titer was determined through ori-ddPCR in duplicates. Particle aggregation was measured with DLS/SLS in the Stunner instrument (Unchained Labs). Endotoxin titer was performed through a LAL assay in the Endosafe® nexgen-PTS™ system (Charles River). Mycoplasma PCR was performed using the Venor®GeM OneStep kit (Minerva Biolabs). The residual endonuclease titer was measured by M-SAN HQ ELISA Kit assay (ArcticZymes Technologies).

Table 4

	Assay	Measurement	Standard deviation
Impurities	Host-cell DNA titer (pg/mL)	1.07E+05	4.07E+04
	Host-cell DNA NGS (% of total DNA)	6.01%	N/A
	Host-cell protein titer (ng/mL)	<37 (LLOQ)	N/A
	Plasmid DNA titer (copies/mL)	4.48E+10	1.22E+10
	Plasmid DNA NGS (% of total DNA)	0.21%	N/A
	Partially filled capsid TEM (%)	16.94%	5.33%
	Partially filled capsid AUC (%)	2.4%	N/A
	Aggregated particles (%)	0%	N/A
	Endotoxin titer (EU/mL)	<1 (LLOQ)	N/A
	Mycoplasma PCR	Negative	N/A
	Residual endonuclease titer (ng/mL)	<0.12 (LLOQ)	N/A

Recommended storage and handling

Thaw the vial at room temperature on the day of the assay. Do not aliquot the sample into other vials, unless it is a natural part of the specific assay. Avoid freeze-thaw cycles. Centrifuge at 8000 rcf for 1 minute every time before opening.

Sequence information

VP1

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQD
DGRGLVLPGYKYLGPFGNLDKGEVNAADAAALEHDKAYD
QQLQAGDNPYLRNHADADEFQERLQEDTSFGGNLGRAVFQA
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GQQPARKRLNFGQTGDESVPDPQLGEPAPPSGVPNT
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VNTEGVYSEPRPIGTRYLTRNL

VP2

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Vector genome sequence (3339 nt)

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