Apertura GENE THERAPY

#1320

Fit-for-Purpose Analytics for Novel Engineered Capsids: Advancing Precision in AAV Characterization

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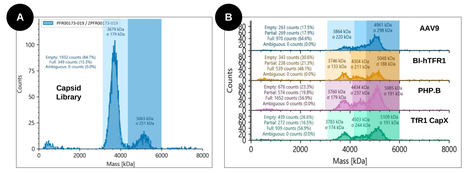
Introduction

Engineered AAV capsids are designed to improve tropism, enhance immune evasion, and increase manufacturability, yet these modifications can introduce unique analytical challenges. Compared to natural serotypes, engineered capsids may exhibit altered physicochemical properties, including reduced thermostability and changes in binding affinity to antibodies used for purification or detection. These differences necessitate the development of fit-for-purpose analytical methods to ensure accurate quantification and characterization of key quality attributes such as genomic titer, capsid titer, and empty/full ratios.

Mass Photometry: Empty/Full Ratio Analysis

Mass photometry provides an efficient and powerful alternative for measuring empty/full capsid ratios. This single-particle method enables direct measurement of AAV capsid populations without requiring antibodies, making it an effective approach for engineered capsids that may have altered binding properties.

Suitability has been confirmed for AAV9, engineered capsids, and capsid libraries (**Fig 1**), demonstrating high precision and reproducibility across multiple operators and production lots. Its implementation strengthens analytical workflows by providing a robust, label-free technique with low volume requirements for evaluating AAV vector quality.



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Capsid	Payload	Full Enrichment	Empty (%)	Partial (%)	Full (%)
Capsid Library	Self-coding cap gene	No enrichment	84.7	-	15.3
AAV9	CEG-hGBA1-HA-WPRE-hGH	Ultracentrifugation	17.5	17.9	64.6
BI-hTFR1	CEG-hGBA1-HA-WPRE-hGH	Ultracentrifugation	30.6	21.3	48.1
PHP.B	CEG-hGBA1-HA-WPRE-hGH	Ultracentrifugation	23.3	19.8	56.9
TfR1 CapX	CEG-hGBA1-HA-WPRE-hGH	Ultracentrifugation	26.6	16.5	56.9

Fig 1. Mass photometry is a suitable analytical technique to measure full:empty ratios of natural AAV serotypes and engineered capsids. **(A)** A library of self-packaging AAV capsids (244,000 variants) was analyzed using the Refeyn SamuxMP mass photometer; no partial capsids were detected. **(B)** AAV9 and three engineered capsids (BI-hTFR1, PHP.B, and TfR1 CapX) were analyzed using the same protocol, confirming the wide utility of the method. **(C)** Tabulated results for empty, partial, and full percentages.

Development of a ddPCR Method Compatible with Natural Serotypes and Engineered Capsids

Engineered capsids may exhibit lower solubility and reduced thermostability compared to natural serotypes, which can introduce challenges in virus dilution and nuclease digestion steps—critical components of ddPCR workflows (**Fig 2**).

To ensure accurate and reproducible genomic titer quantification across both natural and engineered capsids, a refined ddPCR method was developed, which incorporates:

- Using virus formulation buffer (VFB) for sample pre-dilution to mitigate virus aggregation, minimize binding to plastic, and reduce sample matrix effects (**Fig 3**).
- Chemically inhibiting DNase I with EDTA (5mM) to control residual activity (**Fig 4**).

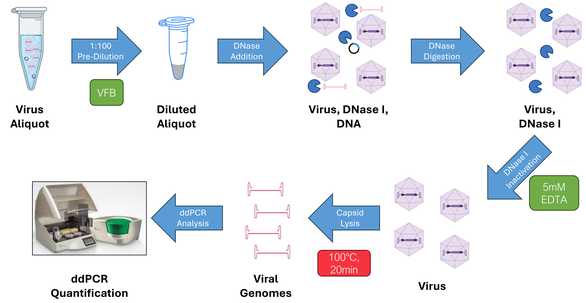

Fig 2. Apertura ddPCR workflow. A virus sample aliquot is pre-diluted 100-fold to minimize aggregation, binding to plastic, and sample matrix effects, then added to a DNase I reaction to digest DNA impurities. The DNase I enzyme is then inactivated. Viral particles are heat-lysed to release DNase-resistant viral genomes, which are then used as template DNA for ddPCR analysis using the Bio-Rad QX200 system.

These optimizations improve assay robustness and reproducibility, enabling precise titer quantification across different capsid variants – both natural AAV serotypes and engineered AAV capsids. The method has been successfully applied to multiple engineered capsids, demonstrating high precision within recommended CV limits (<15%) and suitability for drug product characterization and release.

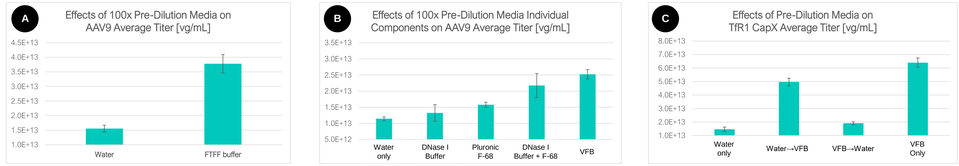

Fig 3. Sample pre-dilution medium affects measured titer. (A) Pre-diluting AAV9 in virus formulation buffer (VFB), which contains salts and Pluronic F-68, results in a higher measured titer compared to pre-diluting AAV9 in water. **(B)** NEB DNase I buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6) and 0.1% Pluronic F-68, individually or together, were used as pre-dilution media and compared to water or VFB when pre-diluting AAV9. DNase I buffer and Pluronic F-68 had an additive effect; when combined, they resulted in a titer comparable to that obtained with VFB. This finding also underscores the importance of using a surfactant like Pluronic F-68 in the DNase I digestion reaction, as previously reported[1]. **(C)** TfR1 CapX was pre-diluted 100x through two serial 10x dilutions, using water, VFB, or combinations thereof. Using VFB as the sole dilution medium minimizes capsid losses and supports virus stability and enhanced assay precision.

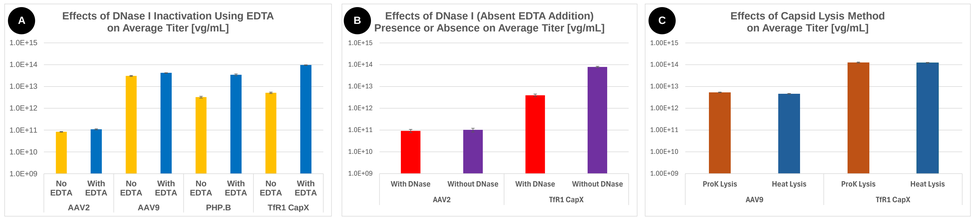

Fig 4. Inactivating DNase I is essential for accurate titer determination of engineered capsids. (A) DNase I temporarily retains residual activity when a temperature increase is used to inactivate it. This residual activity does not appear to significantly overlap with viral genome release from natural AAV serotypes, which have melting temperatures higher than 66°C[2]. However, residual DNase I activity overlaps with genome release from engineered capsids, which may have lower melting temperatures compared to natural serotypes[3]; this results in digestion of a fraction of DNase-resistant viral genomes and undercounting of the true genomic titer. Residual DNase I activity is inhibited by adding EDTA to a final concentration of 5mM post-digestion followed by a 5-minute incubation step at 37°C, after which subsequent heating steps (e.g., heat-based capsid denaturing) may continue. **(B)** Removing DNase I from the ddPCR workflow does not impact the measured titer for AAV2, the natural serotype with the lowest melting temperature[2], but residual DNase I activity significantly lowers the reported titer for engineered AAV capsids like TfR1 CapX. **(C)** The heat-based capsid denaturing method used in Apertura's ddPCR workflow was compared to a Proteinase K capsid digestion method. The two capsid lysis methods yielded comparable results for AAV9 and the TfR1 CapX engineered capsid.

Capsid Titer Assay

Evaluation of a commercially available AAV9 capsid ELISA kit confirmed its suitability for wild-type AAV9 but showed limitations in detecting certain AAV9-based engineered capsids due to reduced binding efficiency (**Fig 5**). To address this gap, an alternative assay is being developed using alternate anti-AAV affinity reagents that offer broader applicability and improved binding across a diverse set of engineered capsids. Initial results indicate strong potential for this assay to provide accurate and reproducible capsid quantification.

				AAV9					TfR1 CapX			
Dilution	Dilution factor	Expected cap/ml	Expected cap/ml x dil factor	Observed cap/ml	Observed cap/ml x dil factor	SD	%CV	% Recovery	Observed cap/ml	Observed cap/ml x dil factor	SD	%CV	% Recovery
D1	1	1.00E+11	1.00E+11	>LOD	>LOD	n/a	n/a	n/a	>LOD	1.67E+07	5.08E+06	30.39%	0.00
D2	4	2.50E+10	1.00E+11	>LOD	>LOD	n/a	n/a	n/a	>LOD	1.29E+07	6.03E+06	46.61%	0.00
D3	16	6.25E+09	1.00E+11	>LOD	>LOD	n/a	n/a	n/a	>LOD	2.44E+07	7.30E+06	29.95%	0.00
D4	64	1.56E+09	1.00E+11	>LOD	>LOD	n/a	n/a	n/a	4.93E+08	1.55E+08	1.46E+07	9.41%	0.00
D5	256	3.91E+08	1.00E+11	3.49E+08	8.94E+10	1.98E+09	2.22%	89.36%	1.34E+08	9.90E+08	9.31E+08	93.97%	0.99%
D6	1024	9.77E+07	1.00E+11	9.40E+07	9.62E+10	9.78E+08	1.02%	96.21%	3.79E+07	2.37E+09	4.91E+08	20.72%	2.37%
D7	4096	2.44E+07	1.00E+11	2.46E+07	1.01E+11	2.48E+09	2.46%	100.57%	1.13E+07	1.46E+10	7.49E+08	5.14%	14.58%
D8	16384	6.10E+06	1.00E+11	7.75E+06	1.27E+11	2.29E+10	18.05%	127.02%	5.29E+06	6.47E+10	2.62E+10	40.55%	64.67%

Fig 5. Evaluation of a commercially available AAV capsid ELISA kit. Prior to testing samples, the capsid titer assay utilizing the PROGEN AAV9 ELISA kit (#PRAAV9) was established with system suitability criteria that included a determined linearity range and inter-assay precision of <10% across analysts (not shown). The kit was then evaluated on AAV9 and the AAV9-based TfR1 CapX capsid; samples were normalized by genomic titer. The ELISA kit was suitable for the analysis of AAV9 virus (AAV9.CEG-hGBA1-HA-WPRE-hGH) and AAV9 empty capsids (not shown). However, the kit was not suitable for the analysis of the TfR1 CapX (same payload), based on the low recoveries observed. This suggests inefficient capsid binding by the kit's anti-AAV antibodies. Development of an alternate ELISA assay using anti-AAV affinity reagents is underway.

Conclusions

The development of optimized analytical workflows compatible with both natural serotypes and engineered capsids ensures robust characterization of AAV vectors.

Analytical methods traditionally used for characterizing natural AAV serotypes must be evaluated to confirm their suitability and applicability for analyzing engineered AAV capsids. Methods such as mass photometry may be readily employed across capsid types. Other methods, like ddPCR and capsid ELISA assays, may require refinements to address engineered capsids' physicochemical properties.

These refinements align with regulatory expectations and enhance the reliability of AAV drug product characterization, supporting clinical translation and manufacturability.