Advancing AAV Process Control with PATsmart™ FlowVPX® Continuous Monitoring and SoloVPE® Orthogonal Validation

Application Note

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Introduction

Accurate quantification of recombinant adeno-associated virus (AAV) genomes is essential for gene therapy safety, efficacy, and consistency. However, downstream process development remains slow, resource-intensive, and dependent on multiple optimization cycles, largely due to the absence of rapid in-process analytics and the long delays of laboratory testing. Current assay methods include ddPCR, ELISA, TEM, AUC, and Gyrolab; emerging methods include HPLC, capillary electrophoresis, dynamic light scattering, and UV-Vis spectroscopy. All methods are either constrained by narrow dynamic ranges, extensive sample preparation, long turnaround times, or low throughput. This underscores the need for robust, manipulation-free assays capable of high-throughput, real-time titer measurement.

To overcome these limitations, we evaluate integration of variable pathlength technology (VPT) with the PATsmart™ FlowVPX® spectrophotometer and KrosFlo® KR2i Real-time Process Management (RPM®) System for continuous, in-line monitoring of AAV concentration during purification, complemented by PATsmart™ SoloVPE® milestone sampling in concentration-diafiltration-concentration (CDC) workflows. The FlowVPX System enables direct nucleic acid quantification during tangential flow filtration (TFF) processes, delivering actionable, real-time data for process control.

This work presents a rapid, reproducible method for AAV titer determination, a critical quality attribute (CQA), specifically designed to enable true in-process monitoring and real-time concentration targeting during AAV manufacturing. The Slope Spectroscopy® method and KrosFlo RPM System demonstrate the capability to not only mitigate batch failure risk, but also support proactive process control for late-stage and commercial manufacturing, while accelerating raw material characterization, reducing development cycles, conserving resources, and shortening manufacturing timelines.

Slope Spectroscopy Method

The Slope Spectroscopy method enhances conventional UV-Vis spectroscopy by deriving concentration from the linear relationship between absorbance and pathlength defined by the Beer-Lambert law.

Linear regression yields an R^2 value, indicating the linearity of up to ten absorbance readings at different pathlengths, verifying compliance with the Beer-Lambert law and ensuring measurement accuracy. The method requires a minimum of five absorbance vs. pathlength data points.

The pathlength can be varied in steps as small as 5 µm on the SoloVPE System and 1 µm on the FlowVPX System, while the two instruments have maximum pathlengths of 15 mm and 3 mm, respectively. This capability captures absorbance behavior across a broad dynamic range. The system then calculates slope-based concentration with a quality threshold of $R^2 \geq 0.999$. Since no dilution or manipulation is required, the method is rapid, precise, reproducible, and nondestructive, enabling efficient AAV quantitation.

AAV Quantitation

To quantify AAV genome and capsid titer, slope-based absorbance measurements are acquired at 260 nm and 280 nm. Nucleic acids (AAV genome) absorb maximally at 260 nm, while capsid proteins absorb at 280 nm. Four extinction coefficients (EC) are necessary to quantify both genome and capsid titer

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simultaneously, as each material has a different EC at each wavelength.

Since spectral overlap exists between capsid proteins and nucleic acids at 260 nm and 280 nm, calculations must account for the absorbance contribution from each substance. Thus, the total slope at each wavelength, m_λ^{full} is the sum of the capsid protein slope m_λ^c and the DNA slope m_λ^d:

$$m_{280}^{full} = m_{280}^c + m_{280}^d$$
$$m_{260}^{full} = m_{260}^c + m_{260}^d$$

According to the Slope Spectroscopy equation, $m = \varepsilon c$, these equations can then be written as:

$$m_{280}^{full} = \varepsilon_{280}^c \cdot conc^c + \varepsilon_{280}^d \cdot conc^d$$
$$m_{260}^{full} = \varepsilon_{260}^c \cdot conc^c + \varepsilon_{260}^d \cdot conc^d$$

Solving these equations for the concentrations $conc^c$ and $conc^d$ results in:

$$conc^d = \frac{R \cdot m_{280} - m_{260}}{\varepsilon_{260}^d \cdot (R \cdot K - 1)} \cdot N_A$$
$$conc^c = \frac{m_{280} - K \cdot m_{260}}{\varepsilon_{280}^c \cdot (1 - R \cdot K)} \cdot N_A$$

Where $R \equiv \frac{\varepsilon_{260}^c}{\varepsilon_{280}^c}$, $K \equiv \frac{\varepsilon_{280}^d}{\varepsilon_{260}^d}$, and N_A is Avogadro's constant. These equations are built into PATsmart™ ViPER® ANLYTX software, the control software for VPT instruments such as the SoloVPE and FlowVPX. The user needs only to enter the genome extinction coefficient and may choose to change the capsid extinction coefficient from the default value, determined experimentally by Sommer, et al.[1]

Materials

- SoloVPE System
  - SoloVPE instrument
  - Cary 60 UV-Vis spectrophotometer
  - ViPER ANLYTX Software
- KrosFlo KR2i RPM System
  - KrosFlo KR2i TFF System
  - FlowVPX instrument
  - Cary 60 UV-Vis Spectrophotometer
  - KrosFlo RPM Software
- Vector product AAV2-GFP

FlowVPX System
The FlowVPX System is a GMP-enabled, in-line UV-Vis spectrophotometer designed for real-time concentration monitoring during processes such as TFF. Using Variable Pathlength Technology (VPT), it adjusts optical pathlengths from 0.001 to 3.000 mm to accurately measure capsid proteins and nucleic acids via A260/A280 ratios. The system offers automated, closed-loop control through open platform communications unified architecture (OPC UA) connectivity, supports 21 CFR Part 11 compliance, and provides rapid, automated analytics that make it a strong PAT tool for gene therapy manufacturing.

KrosFlo KR2i System
The KrosFlo KR2i is a benchtop TFF system for volumes from 10 mL to 10 L, commonly used in AAV purification. It includes a low-shear peristaltic pump, modular filters, automated control modes, and an automatic backpressure valve to maintain stable TMP and reduce membrane fouling.

KrosFlo KR2i RPM System
The KR2i RPM integrates the KR2i TFF system with the FlowVPX spectrophotometer to enable automated, in-line concentration monitoring with real-time endpoint control. Operated through KrosFlo RPM software, the system manages filtration settings and data acquisition, and can run complex TFF workflows using user-defined setpoints informed by data from the VPT system, auxiliary pumps, scales, and backpressure valve.

Experiment Design

AAV2-GFP was selected as a model vector for this proof-of-concept evaluation. The purified AAV2 material was pre-filtered through a 0.2 µm membrane prior to processing, with an initial concentration of 1E+13 vg/mL. The TFF run was conducted as a concentration-diafiltration-concentration (CDC) sequence, incorporating 8 diavolumes (DV) during the diafiltration step (DF). The first and second concentration phases (C1 and C2) each targeted a 2-fold increase, yielding an expected final titer of 4E+13 vg/mL. The process material was filtered after the final concentration for bioburden control and further purified to remove potential aggregates.

Real-time, in-line monitoring of vector concentration was performed using the FlowVPX System to help determine transition points between TFF stages. Samples corresponding to the load, post-C1, post-DF, and post-C2 stages were collected for at-line analysis using the SoloVPE System. In addition to these stages, post-C2 filtered retentate was sampled and analyzed as well, though this material was not monitored in real time using the FlowVPX System. The at-line samples were

subsequently submitted for orthogonal quantification via droplet digital PCR (ddPCR, genome titer) and Gyrolab immunoassay (capsid titer) to compare the analytical methods. Percent differences between methods were calculated relative to the average of the two results.

Reported capsid ECs were 3.72E+6 $M^{-1}cm^{-1}$ at 260 nm and 6.61E+6 $M^{-1}cm^{-1}$ at 280 nm, determined by Sommer, et al.[1] Genome ECs were derived from the single-stranded DNA insert gene coefficient at 260 nm (27 $(mg/mL)^{-1}cm^{-1}$), scaled by genome molecular weight to molar units. Genome ECs at 280 nm were calculated as 0.555 × ε_{260}, consistent with the A260/A280 ratio of 1.8.

At-line measurements were conducted on a single SoloVPE System by two independent analysts. At each process step, sufficient sample volume was collected to prepare three distinct aliquots per analyst, and each aliquot was measured in triplicate. This procedure yielded 18 individual concentration determinations per process step (9 per analyst), providing a structured dataset for evaluating the method's repeatability and intermediate precision.

Table 1. Operating Parameters

System	KR2i RPM System	
Mode	Concentration – Diafiltration – Concentration (CDC)	
Diafiltration volumes	8	
Flow rate	106 mL/min	
Filter	mPES 100 kDa 115 cm^2 (D02-E100-05-S)	
Tubing	Platinum-cured silicone, size #16	
VPT Methods	**FlowVPX**	**SoloVPE**
Sample volume	N/A	100 µL
Starting pathlength	3.0 mm	8.0 mm
Step size	0.5 mm	1.0 mm
Data points	6	6
Averaging time	1.0 s	1.0 s

The methods performed by the SoloVPE and FlowVPX systems were optimized for low absorbance samples, using a fixed slope algorithm for measurement. Details are given in Table 1.

Results

Genome Titer

Comparability across methods was strong. As shown in Table 2, SoloVPE and ddPCR results differed by 20% or less, while FlowVPX and ddPCR results differed by 24% or less. The closest alignment was observed between the SoloVPE and FlowVPX measurements, with differences consistently under 9%.

Intermediate precision testing (Table 3) demonstrated excellent reproducibility for the SoloVPE method, with variation between analysts below 3%. Compared to the ddPCR method, SoloVPE precision was better across all stages of the process (Table 4).

Process monitoring with the FlowVPX instrument provided a complete concentration profile throughout the CDC run (Figure 1), clearly illustrating concentration changes at each stage. Overlaying SoloVPE and ddPCR results with the FlowVPX data confirms overall alignment between the methods. Figure 2 compares concentration measurements at each of the process stages and further reinforces strong correlation across the platforms.

Figure 1 shows concentration decreasing during the diafiltration phase, which is expected as material accumulates on the filter membrane. Toward the end of DF, a short recirculation step occurred prior to C2, during which retained material was released back into the retentate, causing a small, sharp increase in measured titer. This behavior is normal and represents a valuable insight uniquely captured by the FlowVPX System, offering a deeper understanding of what occurs during the process.

Table 2. Analytical Method Comparison, Genome Titer

Stage	Concentration (vg/mL)			|% Difference|		
	ddPCR	SoloVPE	FlowVPX	ddPCR vs. SoloVPE	ddPCR vs. FlowVPX	SoloVPE vs. FlowVPX
Load	1.0E+13	1.1E+13	1.2E+13	9.5%	18%	8.7%
Post-C1	1.8E+13	2.2E+13	2.3E+13	20%	24%	4.4%
Post-DF	2.0E+13	2.2E+13	2.2E+13	9.5%	9.5%	0.0%
Post-C2	4.4E+13	4.6E+13	4.7E+13	4.4%	6.6%	2.2%
Filtered Retentate	4.2E+13	4.2E+13	N/A	0.0%	N/A	N/A

Table 3. SoloVPE Intermediate Precision Results, Genome Titer

Stage	Analyst	SoloVPE Results (vg/mL)	Average (vg/mL)	Standard Deviation (vg/mL)	%RSD
Load	1	1.12E+13	1.13E+13	2.1E+11	1.9%
		1.12E+13			
		1.10E+13			
	2	1.13E+13			
		1.16E+13			
		1.14E+13			
Post-C1	1	2.25E+13	2.18E+13	4.8E+11	2.2%
		2.21E+13			
		2.21E+13			
	2	2.13E+13			
		2.14E+13			
		2.15E+13			
Post-DF	1	2.14E+13	2.16E+13	4.1E+11	1.9%
		2.15E+13			
		2.10E+13			
	2	2.19E+13			
		2.22E+13			
		2.17E+13			
Post-C2	1	4.50E+13	4.56E+13	1.2E+12	2.6%
		4.46E+13			
		4.72E+13			
	2	4.51E+13			
		4.48E+13			
		4.70E+13			
Filtered Retentate	1	4.28E+13	4.24E+13	7.9E+11	1.9%
		4.29E+13			
		4.29E+13			
	2	4.23E+13			
		4.09E+13			
		4.28E+13			

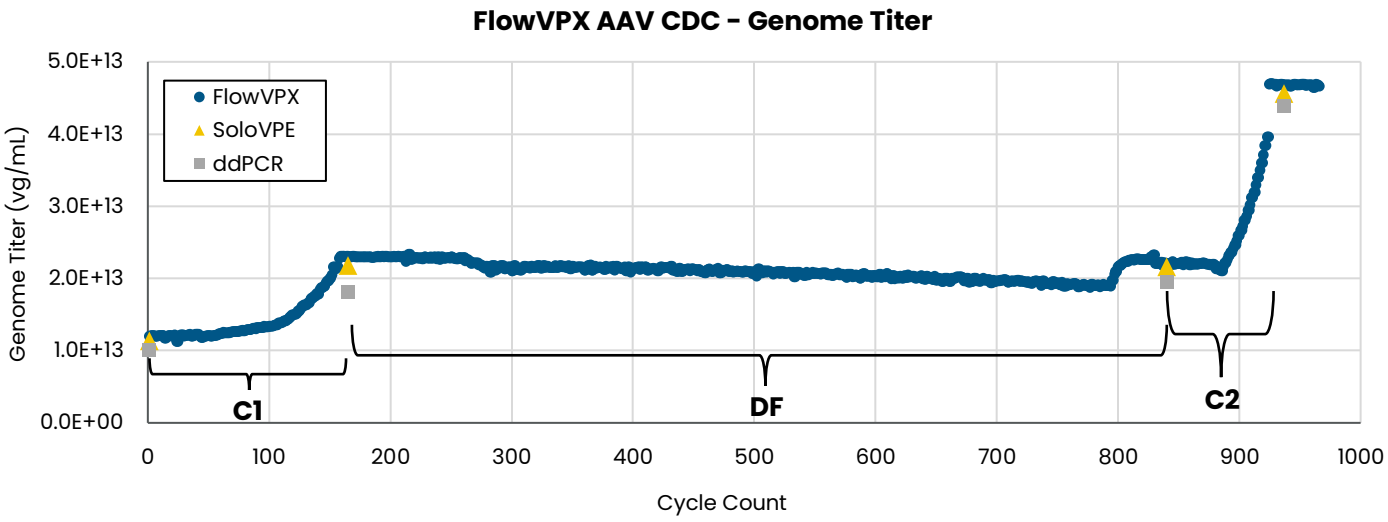

Figure 1. FlowVPX genome titer profile, SoloVPE and ddPCR average measurements overlaid

Table 4. RSD Comparison, Offline Genome Titer

Stage	ddPCR	SoloVPE
Load	3.2%	1.9%
Post-C1	6.5%	2.2%
Post-DF	2.4%	1.9%
Post-C2	2.1%	2.6%
Filtered Retentate	4.3%	1.9%
Average	**3.7%**	**2.1%**

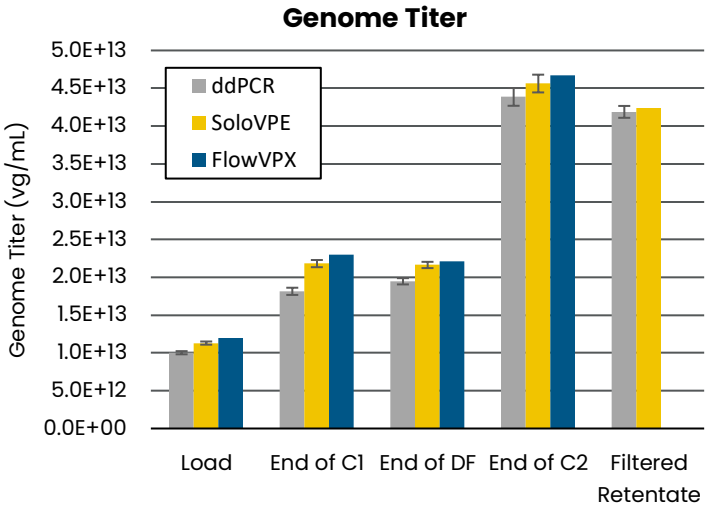

Figure 2. Comparison of genome titer at defined process stages

Capsid Titer

Capsid quantification showed similar trends to those observed for the genome titer. As shown in Table 5, SoloVPE and Gyrolab results differed by up to 22%, while the FlowVPX instrument yielded results within 10% and 11% of the SoloVPE and Gyrolab platforms, respectively.

Intermediate precision remained robust (Table 6) with less than 6% variation between analysts. The repeatability again favored the SoloVPE platform (3.5% average RSD) over the Gyrolab platform (5.2% average RSD) at all stages of the process.

The FlowVPX System also provided continuous, in-line capsid concentration data for the full CDC process (Figure 3). SoloVPE and Gyrolab data are overlaid and align closely with the FlowVPX data. Similar to Figure 1, this graph shows the expected decline in capsid titer as material collects on the membrane. Again, this temporary retention and subsequent release at later stages is characteristic of the process and highlights FlowVPX's ability to capture dynamic changes in real time, offering valuable insight into capsid handling during purification.

Table 5. Analytical Method Comparison, Capsid Titer

Stage	Concentration (cp/mL)			|% Difference|		
	Gyrolab	SoloVPE	FlowVPX	Gyrolab vs. SoloVPE	Gyrolab vs. FlowVPX	SoloVPE vs. FlowVPX
Load	2.1E+13	2.1E+13	2.2E+13	0.0%	4.7%	4.7%
Post-C1	3.6E+13	3.9E+13	3.8E+13	8.0%	5.4%	2.6%
Post-DF	3.3E+13	4.1E+13	3.7E+13	22%	11%	10%
Post-C2	7.2E+13	7.4E+13	7.4E+13	2.7%	2.7%	0.0%
Filtered Retentate	7.1E+13	7.1E+13	N/A	N/A	N/A	N/A

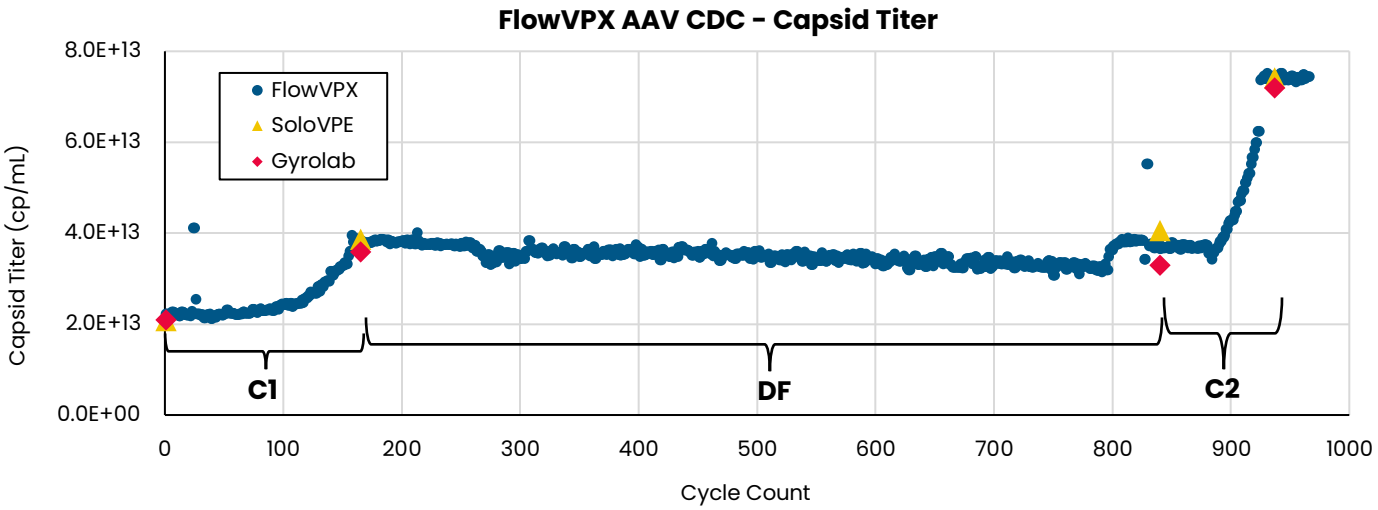

Figure 3. FlowVPX capsid titer profile, SoloVPE and Gyrolab average measurements overlaid

Table 6. SoloVPE Intermediate Precision Results, Capsid Titer

Stage	Analyst	Measured SoloVPE Results (cp/mL)	Average (cp/mL)	Standard Deviation (cp/mL)	%RSD
Load	1	2.08E+13	2.08E+13	4.5E+11	2.2%
		2.04E+13			
		2.10E+13			
	2	2.10E+13			
		2.15E+13			
		2.03E+13			
Post-C1	1	3.78E+13	3.86E+13	2.1E+12	5.4%
		4.26E+13			
		3.68E+13			
	2	3.79E+13			
		3.91E+13			
		3.77E+13			
Post-DF	1	4.12E+13	4.06E+13	2.0E+12	4.9%
		4.17E+13			
		4.02E+13			
	2	4.01E+13			
		4.31E+13			
		3.72E+13			
Post-C2	1	7.66E+13	7.42E+13	2.6E+12	3.5%
		7.18E+13			
		7.44E+13			
	2	7.19E+13			
		7.25E+13			
		7.79E+13			
Filtered Retentate	1	7.19E+13	7.05E+13	1.1E+12	1.6%
		7.09E+13			
		7.07E+13			
	2	6.85E+13			
		7.05E+13			
		7.05E+13			

Table 7. RSD Comparison, Offline Capsid Titer

Stage	Gyrolab	SoloVPE
Load	13%	2.2%
Post-C1	1.5%	5.4%
Post-DF	7.3%	4.9%
Post-C2	2.6%	3.5%
Filtered Retentate	1.6%	1.6%
Average	**5.2%**	**3.5%**

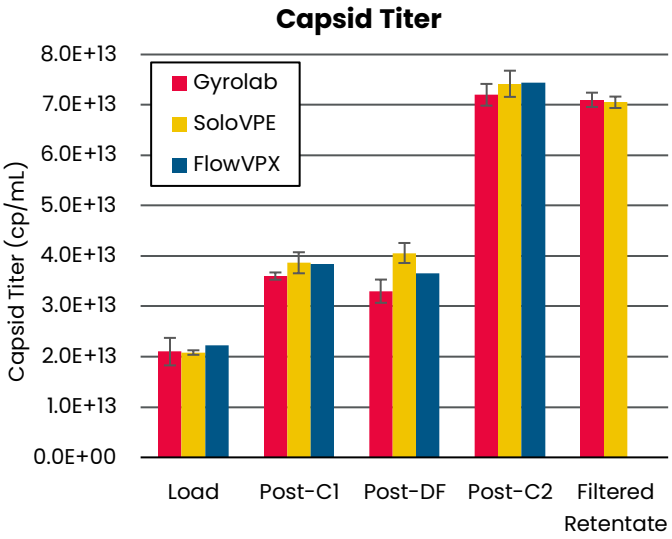

Figure 4. Comparison of capsid titer at defined process stages

Conclusion

The KrosFlo RPM System enables real-time capture of dynamic changes throughout AAV concentration workflows, providing critical insights into TFF process behavior. Continuous, in-line monitoring by the FlowVPX instrument generates a detailed concentration profile across each stage of the CDC process, allowing detection of transient events such as membrane interactions or material retention that are often missed by endpoint assays. By delivering immediate feedback, the FlowVPX System enhances process understanding and supports proactive decision-making during both process development and manufacturing. While this work demonstrates the utility of the platform for monitoring purified material during TFF, broader applicability across earlier or less-purified process streams warrants further evaluation.

Complementing this, the SoloVPE System delivers rapid, slope-based absorbance measurements at 260 nm and 280 nm, enabling samples to be withdrawn from the process stream and analyzed within minutes.

In contrast, the ddPCR method can be a costly, time-consuming approach for AAV quantification, with consumables and reagents totaling up to $1000 per plate. The workflow typically requires 24 to 48 hours from sample preparation through data analysis, significantly delaying feedback. Additionally, ddPCR assays can fail due to factors such as inhibition, droplet generation issues, or amplification variability, requiring the entire assay to be repeated.

Additionally, maintaining consistency across ddPCR analysts requires training and strict procedural controls. By comparison, intermediate precision is virtually nonexistent when using the SoloVPE and KrosFlo RPM Systems, as measurements are highly reproducible and require minimal operator intervention.

Together, the FlowVPX and SoloVPE Systems provide a fast, cost-effective, and highly reproducible analytical framework that delivers real-time AAV genome and capsid titer quantification as well as exclusive process insights. The variable pathlength platform and method strengthen confidence in quantification while enhancing overall process understanding control.

Reference

1. Sommer, J.M., Smith, P.H., Parthasarathy, S., Isaacs, J., Vijay, S., Kieran, J., Powell, S.K., McClelland, A., & Wright, J.F. (2003). Quantification of adeno-associated virus particles and empty capsids by optical density measurement. Molecular therapy: The journal of the American Society of Gene Therapy. https://pubmed.ncbi.nlm.nih.gov/12573625/

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