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Instruments

Rapid Monitoring of AAV Production Kinetics with Multi-Attribute Assays *on the Leo Simple Western System*

Highlights

- The Leo™ System powered by Simple Western™ technology allows for the monitoring of multiple critical AAV attributes in a single workflow.
- Leo measures Rep expression, capsid titer, and empty/full capsid ratio at the cell culture stage by leveraging PROGEN® rAAV2 Rep proteins, antibodies, AAV standard material and AAV empty capsids to generate standard curves and controls.
- Rep protein expression peaks at 48 hours post-transfection, indicating maximum intracellular AAV production activity.
- Capsid production correlates with Rep protein expression with maximal capsid per cell production efficiency at 48 hours, then plateaus.
- Capsid accumulation in the supernatant continues through 72 hours, despite a sharp decline in Rep expression, indicating cell lysis and passive release/leakage.
- Packaging efficiency increases over time, reaching ~30% full capsids between 24 and 48 hours, then declines gradually at 72 hours.
- This workflow can help identify the optimal AAV harvest window; in this study, 48 hours if harvesting from cells only, 72 hours if harvesting from cells and supernatant.

Why This Workflow Matters for AAV Production

Adeno-associated virus (AAV) manufacturing requires careful monitoring of several critical quality and process attributes, including viral productivity, capsid formation, and genome packaging efficiency. These attributes are typically assessed using a combination of orthogonal assays, such as ELISA, qPCR, and Western blotting, each requiring separate workflows and significant hands-on time.

By enabling automated quantification of Rep proteins, capsid titer, and empty/full capsid ratio within a single platform with minimal sample preparation at the cell culture stage, the Leo system provides an approach that allows researchers to monitor AAV production kinetics in a more integrated and efficient manner, enabling faster identification of optimal process conditions and deeper understanding of production dynamics.

Case Study

Evaluating Multiple CQAs of AAV5 Production Across Key Timepoints

Rep protein expression (intracellular production activity), capsid titer (viral particle yield), and empty/full capsid ratio (packaging efficiency) were measured in AAV5 production samples collected at multiple timepoints during upstream processing. Cell lysates and corresponding supernatants were analyzed sequentially on the Leo system with standard curves and control samples to establish absolute quantification of these CQAs.

All assays demonstrated robust nonlinear fit, accuracy, and reproducibility across their respective dynamic ranges, as confirmed by standard curve and control recoveries.

Details on materials and methods are provided in Appendix 1.

Results

Rep protein expression peaks at 48 hours, signaling maximum intracellular production activity.

Rep proteins were quantified in both cell lysates and supernatants to assess intracellular production activity over time. All four Rep isoforms showed a consistent temporal profile, with expression increasing from 24 to 48 hours post-transfection and declining sharply by 72 hours in cell lysates (Figure 1A). The highest intracellular concentrations were observed at 48 hours, with Rep52 increasing from 45.5 µg/mL at 24 hours to 96.3 µg/mL at 48 hours, before dropping to 4.6 µg/mL at 72 hours (Table 1). Similar trends were observed for Rep40, Rep68, and Rep78. Minimal Rep protein signal was detected in supernatant samples, consistent with the intracellular role of Rep proteins. These results indicate that intracellular viral production activity peaks at 48 hours and declines substantially thereafter.

FIGURE 1

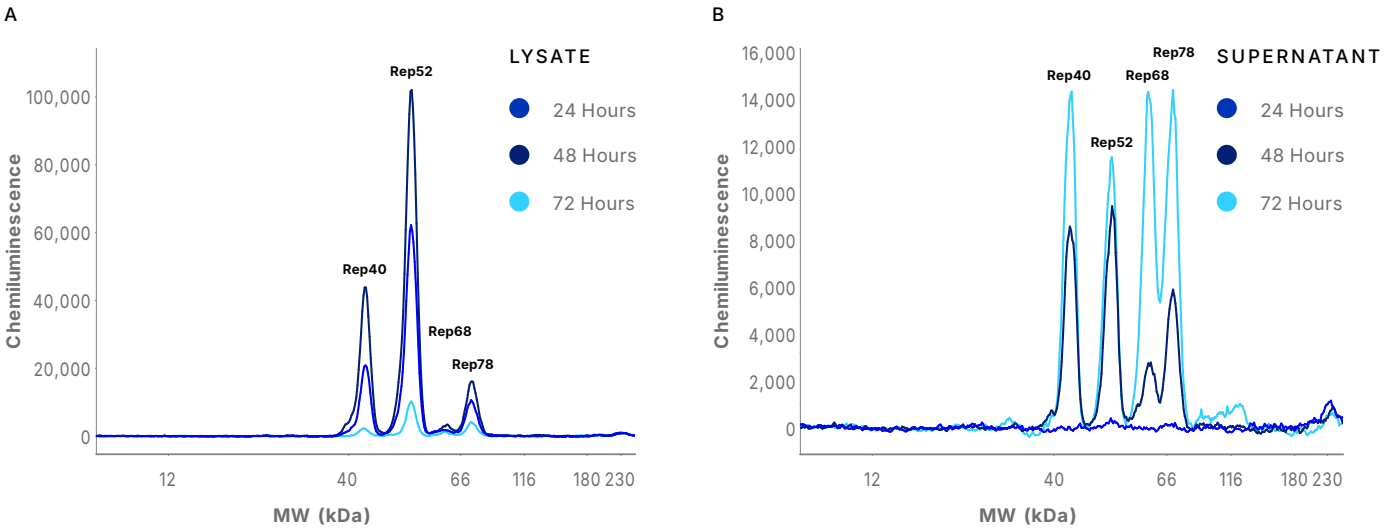

Figure 1. Rep protein expression peaks at 48 hours, identifying the window of maximum intracellular AAV production activity. (A) Representative electropherograms of Rep40, Rep52, Rep68, and Rep78 in cell lysates collected at 24, 48, and 72 hours post-transfection show increasing expression from 24 to 48 hours followed by a marked decline at 72 hours. (B) Electropherograms of corresponding supernatant samples show minimal levels of all Rep isoforms across timepoints, consistent with the intracellular role of Rep proteins.

TABLE 1

Rep (µg/mL)	Time (Hours)	Rep40	Rep52	Rep68	Rep78
Lysate	24h	19.1	45.5	1.1	8.4
	48h	49.8	96.3	2.2	15.9
	72h	1.9	4.6	0.4	2.6
Supernatant	24h	-	-	-	-
	48h	0.020	0.014	0.003	0.010
	72h	0.030	0.019	0.013	0.028

Table 1. Quantitative analysis of Rep expression confirms peak intracellular levels at 48 hours, with all isoforms exhibiting a similar temporal profile. Rep protein concentrations decline sharply by 72 hours, indicating reduced intracellular production activity at later timepoints.

Intracellular Capsid Titer Peaks at 48 Hours and Continues to Accumulate Extracellularly Through 72 Hours

Capsid titer analysis was performed to quantify total viral particle production in both lysate and supernatant samples (Figures 2A and 2B). This enables differentiation between intracellular production and extracellular accumulation of viral particles over time. Intracellular capsid titers increased markedly between 24 and 48 hours, reaching an average of 6.4×10^{13} VP/mL at 48 hours, compared with 2.0×10^{13} VP/mL at 24 hours (Table 2). While intracellular titers remained high at 72 hours, the rate of increase plateaued. In contrast, capsid titers in the supernatant continued to rise through 72 hours, increasing from undetectable levels at 24 hours to 1.4×10^{11} VP/mL at 48 hours and 2.0×10^{12} VP/mL at 72 hours (Table 2). This continued increase reflects accumulation of previously produced viral particles in the extracellular environment due to cell lysis and passive release/leakage.

FIGURE 2

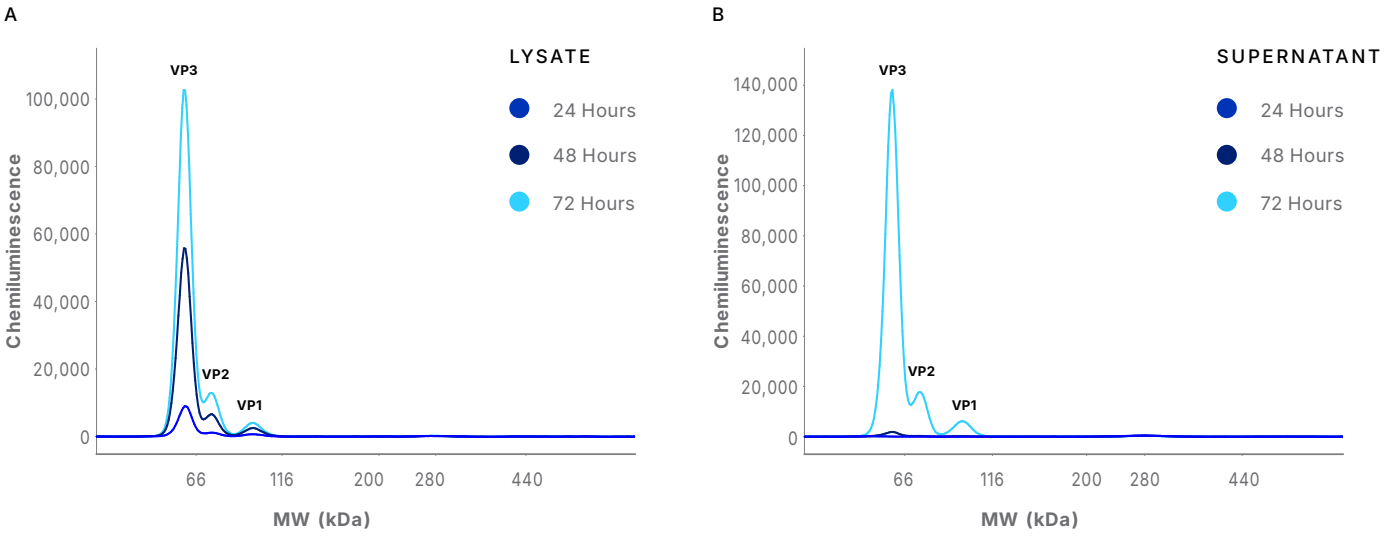

Figure 2. Capsid titer profiles distinguish peak intracellular production from continued extracellular accumulation during AAV5 production. (A) Representative electropherograms of AAV5 capsid proteins (VP1, VP2, and VP3) in cell lysates collected at 24, 48, and 72 hours post-transfection show a marked increase in intracellular capsid levels from 24 to 48 hours, indicating peak viral particle production at 48 hours (measured titers were dilution corrected to lysate concentration). (B) Electropherograms of corresponding supernatant samples with no capsid signal at 24 hours, with a substantial increase from 48 to 72 hours, reflecting progressive accumulation of viral particles in the extracellular environment.

TABLE 2

VP/mL	Time (Hours)	Run 1	Run 2	Run 3	Average	% CV
Lysate	24h	1.8×10^{13}	2.0×10^{13}	2.1×10^{13}	2.0×10^{13}	15.8 (n=15)
	48h	5.9×10^{13}	6.8×10^{13}	6.7×10^{13}	6.4×10^{13}	12.2 (n=11)
	72h	5.0×10^{13}	6.1×10^{13}	5.5×10^{13}	5.5×10^{13}	13.1 (n=9)
Supernatant	24h	-	-	-	-	-
	48h	1.5×10^{11}	1.4×10^{11}	1.2×10^{11}	1.4×10^{11}	16.0 (n=16)
	72h	2.2×10^{12}	2.2×10^{12}	1.7×10^{12}	2.0×10^{12}	14.4 (n=11)

Table 2. Capsid titer quantification across all timepoints, confirming maximal intracellular titers at 48 hours and continued increases in supernatant titers through 72 hours. Together, these data demonstrate that intracellular capsid production is most active by 48 hours, while later increases in extracellular titers primarily reflect particle release and accumulation. % CV values demonstrate reproducible measurements across independent runs.

Capsid Production Per Cell Increases Significantly from 24 to 48 Hours

Productivity peaked at 48 hours, with lysate samples producing approximately 2.1×10^6 VP per cell, compared with 6.5×10^5 VP per cell at 24 hours (Table 3). While VP/cell remained high at 72 hours (~2.3×10^6), this increase occurred alongside a substantial decline in Rep expression, indicating that later timepoints reflect continued accumulation and release of previously synthesized particles rather than sustained intracellular production. These results suggest that 48 hours corresponds to maximum active capsid production.

TABLE 3

VP/cell	Time (Hours)	Run 1	Run 2	Run 3	Average	% CV
Lysate	24h	6.0×10^5	6.6×10^5	7.0×10^5	6.5×10^5	15.8 (n=15)
	48h	2.0×10^6	2.3×10^6	2.2×10^6	2.1×10^6	12.2 (n=11)
	72h	2.1×10^6	2.6×10^6	2.3×10^6	2.3×10^6	13.1 (n=9)

Table 3. Capsid production per cell increases from 24 to 48 hours and remains elevated at later timepoints. Quantification of viral particles per cell (VP/cell) in lysate samples shows a substantial increase from 24 to 48 hours post-transfection. While VP/cell values continue to rise slightly at 72 hours, this occurs concurrently with a decline in Rep expression, indicating that the later timepoint reflects continued accumulation or release of previously synthesized particles rather than sustained intracellular production activity. % CV values demonstrate reproducible measurements across independent runs.

Packaging Efficiency Peaks at 48 Hours

To assess product quality, the proportion of full capsids was measured across time points. Because capsid assembly and genome packaging are distinct processes, measuring empty/full capsid ratio provides insight into encapsulation efficiency.

In lysate samples, the percentage of full capsids increased from 12.1% at 24 hours to 30.3% at 48 hours, before decreasing slightly to 27.7% at 72 hours. Supernatant samples showed a similar trend, with % full reaching 35.4% at 48 hours and declining to 26.2% at 72 hours (Figure 3, Table 4). These results demonstrate that genome packaging efficiency improves during early production but does not continue to increase at later timepoints, despite ongoing capsid accumulation.

FIGURE 3

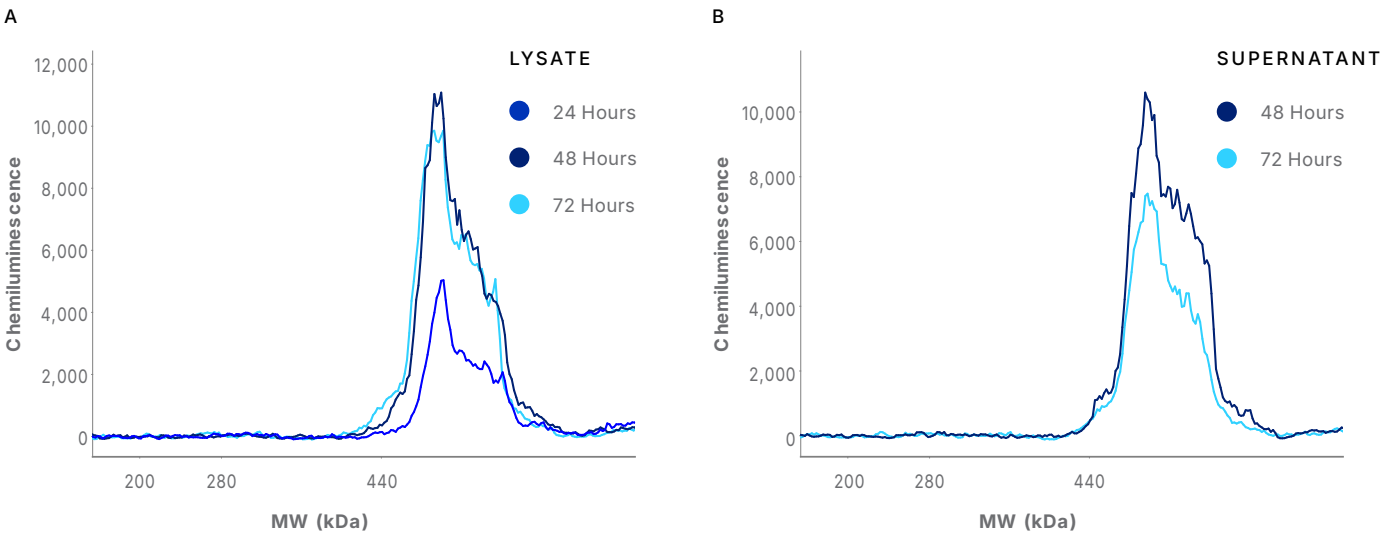

Figure 3. Maximum packaging efficiency is observed at 48 hours and does not improve at later timepoints despite continued capsid accumulation. (A) Representative electropherograms of encapsulated DNA in AAV5 production samples show increasing signal from 24 to 48 hours in lysates, followed by a slight decrease at 72 hours. (B) Supernatant samples exhibit a similar trend, with peak signal at 48 hours and reduced intensity at 72 hours.

TABLE 4

% Full	Time (Hours)	Run 1* (n=6)	Run 2* (n=3)	Run 3* (n=3)	Average	% CV (n=12)
Lysate	24h	13.2	11.4	10.7	12.1	10.9
	48h	32.7	27.3	28.3	30.3	9.0
	72h	31.0	23.0	26.0	27.7	13.1
Supernatant	24h	-	-	-	-	-
	48h	34.8	32.7	39.3	35.4	7.9
	72h	24.9	23.7	31.4	26.2	12.7

Table 4. Summarizes the percentage of full capsids across all timepoints, confirming that packaging efficiency increased from 12.1% to 30.3% in lysates and reached 35.4% in supernatants at 48 hours before declining at 72 hours. Together, these data indicate that genome packaging efficiency peaks at 48 hours and does not improve further at later harvest times. *Each run represents an independent empty/full capsid determination using an independently measured capsid titer. % CV values demonstrate reproducible measurements across independent runs.

Integrated Multi-Attribute Analysis Identifies 48 Hours as the Optimal Harvest Window

By combining measurements of Rep expression, capsid titer, and empty/full capsid ratio, a comprehensive view of AAV production kinetics was obtained. Across all three attributes, 48 hours post-transfection represents the optimal balance of:

- Maximum intracellular production activity (Rep expression)
- Peak capsid production (lysate titer)
- Highest packaging efficiency (% full capsids)

Although capsid accumulation in the supernatant continues through 72 hours, the marked decline in Rep expression indicates that cells are no longer actively producing virus at later timepoints. Instead, this phase reflects release of previously generated particles.

Collectively, these results demonstrate that multi-attribute analysis provides a mechanistic basis for defining optimal harvest timing, with 48 hours identified as the most productive and efficient timepoint for AAV5 production.

Takeaways

- Multi-attribute analysis enables simultaneous monitoring of AAV production activity, viral yield, and packaging efficiency within a single platform, reducing reliance on multiple orthogonal assays.
- Rep expression provides a direct indicator of intracellular production activity, revealing that AAV5 production is most active at 48 hours post-transfection.
- Capsid titer measurements distinguish intracellular production from extracellular accumulation, showing that continued increases in supernatant titers at later timepoints reflect particle release rather than sustained production.

Takeaways *(continued)*

- Packaging efficiency peaks at 48 hours and does not improve at later timepoints, highlighting the importance of monitoring genome encapsulation alongside capsid production.
- Integrated analysis of these attributes identifies 48 hours as the optimal harvest window, providing the best balance of productivity, yield, and product quality.
- By enabling the quantification of rep proteins, total capsid titer, and empty/full capsid ratios on the Leo System powered by Simple Western technology, this workflow significantly streamlines AAV development and can be utilized in both upstream and downstream processes.

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Appendix 1

TABLE A1

Materials Used in this Study

Material	Vendor	Catalog #
Leo System	R&D Systems	004-550
Simple Western Leo 12–230 kDa Separation Module		SWSM-W014
Simple Western Leo 66–440 kDa Separation Module		SWSM-W016
Rb anti-dsDNA mAb		NBP3-07302
Anti-Rabbit Detection Module		DM-001
Anti-Mouse Detection Module		DM-002
Goat Anti-Mouse HRP Secondary Antibody		040-655
RIPA Lysis Buffer		040-483
DMSO Inhibitor Mix		040-510
Aqueous Inhibitor Mix		040-482
Recombinant S. marcescens NucA nuclease Protein, CF		10038-NAB
AAV2 Rep40	PROGEN	640846
AAV2 Rep52		640847
AAV2 Rep68		640832
AAV2 Rep78		640848
anti-AAV2 Rep mouse monoclonal, 259.5		61071
anti-AAV VP1/VP2/VP3 mouse monoclonal, B1		690058
AAV5 empty capsids		66V050
AAV5 standard material (eGFP)		66V051
DNase I Reaction Buffer	NEB	B0303S
Poloxamer 188 Solution (PF-68)	Sigma Aldrich	P5556
BCA Protein Assay	Thermo Fisher Scientific	23225

Methods

AAV5 Production Samples

AAV5 production samples were obtained from transiently transfected HEK293 cells (PROGEN). Cell pellets and corresponding supernatants were collected at 24, 48, and 72 hours post-transfection. Cell pellets were harvested at 7.5×10^7 cells (24 and 48 hours) and 6.0×10^7 cells (72 hours).

Cell pellets were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors according to an in-house cell lysis protocol. Lysates were clarified and protein concentrations were determined using a BCA assay prior to analysis.

Rep Quantification

Rep expression was quantified on the Leo system with a 12–230 kDa separation module and a 30-minute separation method. Samples were diluted to the desired concentrations in 1X Master Mix and denatured under reducing conditions at 95 °C for 5 minutes. Mouse anti-AAV2 Rep monoclonal antibody (PROGEN) was used at 20 µg/mL in Antibody Diluent 2, followed by incubation with an anti-mouse HRP secondary antibody. Multiplexed standard curves were generated using recombinant Rep40, Rep52, Rep68, and Rep78 proteins (PROGEN) across an 8-point, 2-fold dilution series. Quality control samples, including high and low controls, were included to assess assay performance. All measurements were performed using multi-point dilution series of the samples to ensure quantification within the standard curve range.

Capsid Titer Determination

Capsid titer was quantified using the Leo system with a 66–440 kDa separation module under default conditions. Samples were diluted to 1.25X the final concentration in PBS containing 0.001% PF-68 and mixed with 5X Master Mix (MM), 4-parts sample and 1-part 5X MM, prior to denaturation at 95 °C for 5 minutes. Capsid proteins (VP1, VP2, and VP3) were detected using a mouse anti-AAV VP1/VP2/VP3 monoclonal antibody (clone B1, PROGEN) at 20 µg/mL, followed by incubation with a goat anti-mouse HRP secondary antibody at 1X in Antibody Diluent 2. Standard curves were generated using AAV5 standard material from PROGEN across an 8-point, 2-fold dilution series. Quality control samples, including high and low controls, were included to assess assay performance. All measurements were performed using multi-point dilution series of the samples to ensure quantification within the standard curve range.

Empty/Full Capsid Ratio Determination

The proportion of genome-containing (full) capsids was determined using a DNA-based Simple Western assay on the Leo system with a 66-440 kDa separation module under default conditions. Standards, controls, and samples, at 1.25X the final concentration in PBS containing 0.001% PF-68, were pre-treated with 10 µg/mL NucA and 1X DNase I buffer for 1 hour at room temperature to degrade unencapsulated DNA. Enzymatic activity was stopped by adding EDTA to 50 mM final. Samples were then mixed with pre-denatured Master Mix (95 C for 5 minutes, cooled on ice) without heat treatment prior to separation. Encapsulated DNA was detected using a rabbit anti-dsDNA monoclonal antibody, followed by an anti-rabbit HRP secondary antibody. Standard curves and controls were prepared using defined mixtures of full and empty AAV5 capsids (PROGEN) at 100% (full only), 85%/15%, 70%/30%, 55%/45%, 40%/60%, 25%/75%, 10%/90%, 0% (empty only). Full standard material determines the top % full standard. Standard, controls, and samples were loaded at the same capsid titer, 3×10^9 VP/mL, enabling quantification of percent full capsids across samples.

Data Analysis

All data were analyzed using Compass for Simple Western software (version 7.1). Standard curves were fitted using log–log nonlinear regression with $1/y^2$ weighting. Concentrations of unknown samples were interpolated from corresponding standard curves and corrected for dilution factors. Assay accuracy was assessed using percent recovery of standards and control samples, with acceptable recovery defined as 80–120%. Because the Leo system uses up to 4 x 25 capillary cartridges for a maximum of 100 capillaries per run, a reference standard was included across all cartridges to enable cartridge correction during analysis and ensure consistent absolute quantification for each respective assay.

Appendix 2: Assay Performance

Rep Assay Performance

Multiplexed standard curves were generated using recombinant Rep40, Rep52, Rep68, and Rep78 proteins across an 8-point, 2-fold dilution series. Representative electropherograms demonstrate clear separation of all four Rep isoforms and concentration-dependent increases in signal intensity across the tested range (Figure A2.1, Table A2.1).

FIGURE A2.1

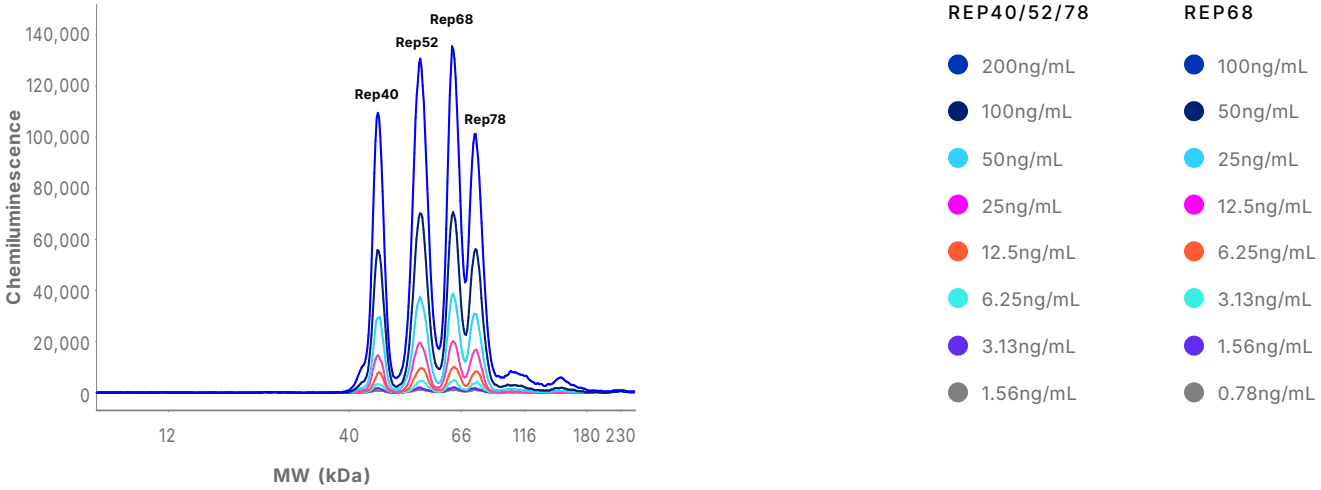

Figure A2.1. All four Rep proteins exhibited strong titration across their respective concentration ranges. Standard curve recoveries remained within the accepted 80–120% range for all the concentrations tested, with recoveries ranging from 86.7% to 114.9%. High- and low-control samples also demonstrated acceptable recoveries across all four isoforms, confirming assay accuracy throughout the dynamic range.

TABLE A2.1

rRep		% Recovery (n=2)			
		Rep40	Rep52	Rep68	Rep78
Standard Curve	200ng/mL	104.5	101.2	-	98.0
	100ng/mL	97.4	97.1	99.2	97.3
	50ng/mL	103.1	104.3	95.9	104.8
	25ng/mL	102.4	106.3	102.4	105.1
	12.5ng/mL	98.4	101.0	107.8	102.8
	6.25ng/mL	92.9	92.4	105.2	95.5
	3.13ng/mL	92.5	86.7	94.3	91.9
	1.56ng/mL	111.4	114.9	90.7	106.8
	0.78ng/mL	-	-	107.7	-
Controls	HC	104.6	102.1	99.8	99.1
	LC	115.0	108.5	101.1	106.3

Table A2.1. Percent recoveries for recombinant Rep40, Rep52, Rep68, and Rep78 standards and controls. All standard and control recoveries fell within the accepted 80–120% range, supporting accurate quantification of Rep expression in production samples. Standards, controls, and reference standard were run in duplicate.

Capsid Titer Assay Performance

Capsid titer assay performance was evaluated using an AAV5 standard material across an 8-point dilution series ranging from 2.00×10^{11} to 4.87×10^{9} VP/mL. Representative electropherograms show concentration-dependent increases in VP1, VP2, and VP3 signal intensity across the tested range (Figure A2.2, Table A2.2)

FIGURE A2.2

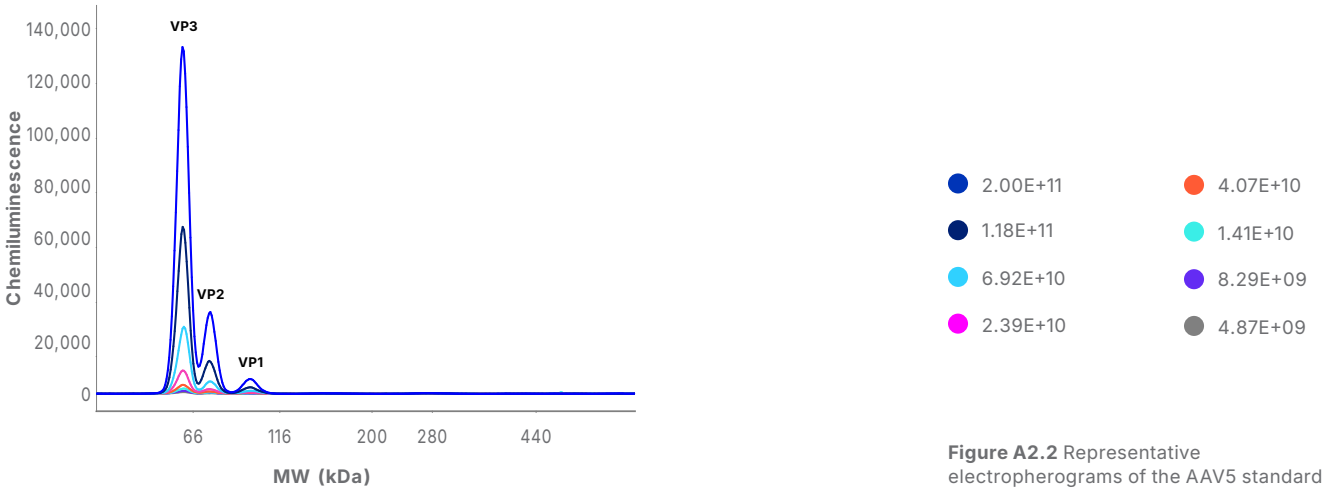

Figure A2.2 Representative electropherograms of the AAV5 standard material show concentration-dependent increases in VP1, VP2, and VP3 signal intensity across an 8-point dilution series. Regression analysis performed across three independent runs demonstrated strong assay reproducibility (average R^2 = 0.9823; average slope = 1.49).

n=3	R2	Slope
Average	0.9823	1.49
%CV	1.9	2.1

TABLE A2.2

Standards (VP/mL)		Run 1	Run 2	Run 3	Average	%CV (n=6)
Standard Curve	2.00×10^{11}	96.0	115.9	99.8	103.9	9.7
	1.18×10^{11}	110.2	113.3	107.6	110.4	5.4
	6.92×10^{10}	112.0	98.2	98.7	103.0	7.6
	2.39×10^{10}	98.3	91.3	106.7	98.7	10.5
	4.07×10^{10}	89.1	81.1	100.8	90.4	10.4
	1.41×10^{10}	87.4	86.6	80.1	84.7	5.7
	8.29×10^{9}	99.2	101.9	96.7	99.3	4.4
	4.87×10^{9}	113.8	122.9	115.6	117.4	9.6
Controls	HC	105.6	118.3	104.8	109.6	8.1
	LC	100.2	97.0	92.2	96.5	8.7

Table A2.2. Standard and control recovery data are summarized, where all but one standard replicates had recoveries falling within accepted performance criteria across the assay range. Together, these results support robust and reproducible quantification of AAV5 capsid titers. Standards, controls, and reference standard were run in duplicate in each run.

Empty/Full Capsid Ratio Assay Performance

Assay performance for empty/full capsid ratio determination was assessed using defined mixtures of full and empty AAV5 capsids The standard curve for this study spanning 0–79% full capsids demonstrated concentration-dependent increases in encapsulated DNA signal and robust regression performance across independently executed runs (Figure A2.3, Table A2.3).

FIGURE A2.3

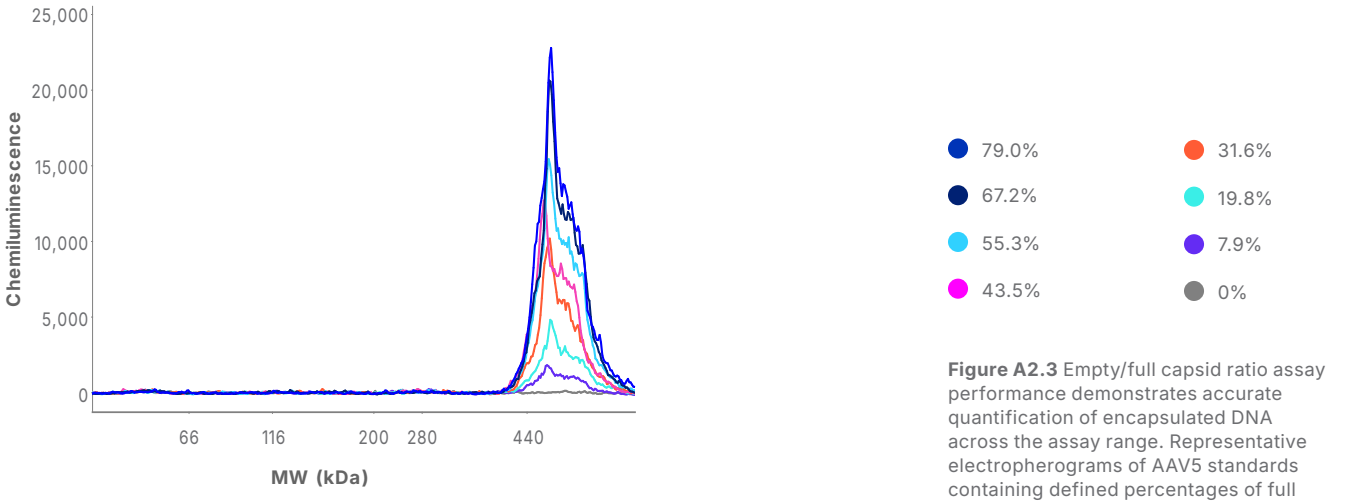

Figure A2.3 Empty/full capsid ratio assay performance demonstrates accurate quantification of encapsulated DNA across the assay range. Representative electropherograms of AAV5 standards containing defined percentages of full capsids show concentration-dependent increases in encapsulated DNA signal across the tested range (0–79% full capsids). Regression analysis performed across two independent runs demonstrated excellent assay reproducibility (average R^2 = 0.9965; average slope = 1.068).

n=2	R2	Slope
Average	0.9965	1.068
%CV	0.03	2.8

TABLE A2.3

Standards (% Full)		Run 1	Run 2	Average	%CV (n=8)
Standard Curve	79.0%	96.8	95.2	96.0	5.0
	67.2%	99.1	98.6	98.8	3.1
	55.3%	102.7	101.2	102.0	6.9
	43.5%	103.7	100.9	102.3	4.9
	31.6%	99.8	108.8	104.3	5.1
	19.8%	99.5	98.7	99.1	4.2
	7.9%	99.4	97.8	98.6	4.0
Controls	HC	99.7	99.7	101.3	3.3
	LC	99.4	99.4	95.4	6.7

Table A2.3. Standard and control recovery data are summarized, with recoveries falling within accepted performance criteria across the assay range. Together, these results support robust and reproducible quantification of capsid packaging efficiency. Standards, controls, and reference standard were run in quadruplicate in each run.