Advanced analytical methods optimize rAAV process development outcomes and improve yields and full particle ratios

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Introduction

Recombinant adeno-associated viruses (rAAVs) are the leading vehicles for gene therapy, but large-scale manufacturing that balances high yield and quality remains a major challenge. A key difficulty is minimizing empty particles while maximizing full capsids containing the gene of interest. To address this, we employed mass photometry (MP) as a rapid and precise method to distinguish empty, partial, and full rAAVs based on their mass distribution[1]. We optimized upstream production parameters and downstream purification using MP, identifying optimal transfection conditions that yielded the highest proportion of full particles and improving purification by selecting fractions enriched in full capsids. Nanopore sequencing[2] further confirmed the identity and purity of rAAV preparations, revealing differences in genome heterogeneity. Our findings demonstrate the critical role of advanced analytics in optimizing rAAV production and enhancing gene therapy manufacturing.

Mass Photometry Samux^{MP} (Refeyn)

Nanopore Sequencing Mk1C (Oxford Nanopore Technologies)

Results and Discussion

1 MP evaluates USP parameters

To optimize the genome titer and full-to-empty capsid ratio in rAAV5 production, we investigated key upstream parameters in a triple transfection process using HEK293 cells grown in suspension. Cells were transfected with the same plasmids for the gene of interest (pGOI) and the rAAV5 capsid (pRep2Cap5), but either pHelper A or pHelper B was used. Two stoichiometries were tested: a 1:1:1 and a 2:1:0.5 ratio of pRep2Cap5:pGOI:pHelper. Following an optimized clarification process, vector genome titers were quantified by ddPCR, and the percentage of full rAAV particles was determined using mass photometry (SamuxMP).

Table 1: Overview of the experimental conditions, vector genome titers and % full particles in the clarified harvests.

Condition No.	Plasmid ratio (pRep2Cap5:pGOI:pHelper)	pHelper	% Full	Titer ddPCR (vg/ml)	Total VGs
1	1:1:1	pHelper A	8.8%	2.49e+11	7.47e+12
2	2:1:0.5	pHelper A	8.4%	3.39e+11	1.02e+13
3	1:1:1	pHelper B	16.5%	2.18e+11	6.54e+12
4	2:1:0.5	pHelper B	8.2%	2.02e+11	6.06e+12

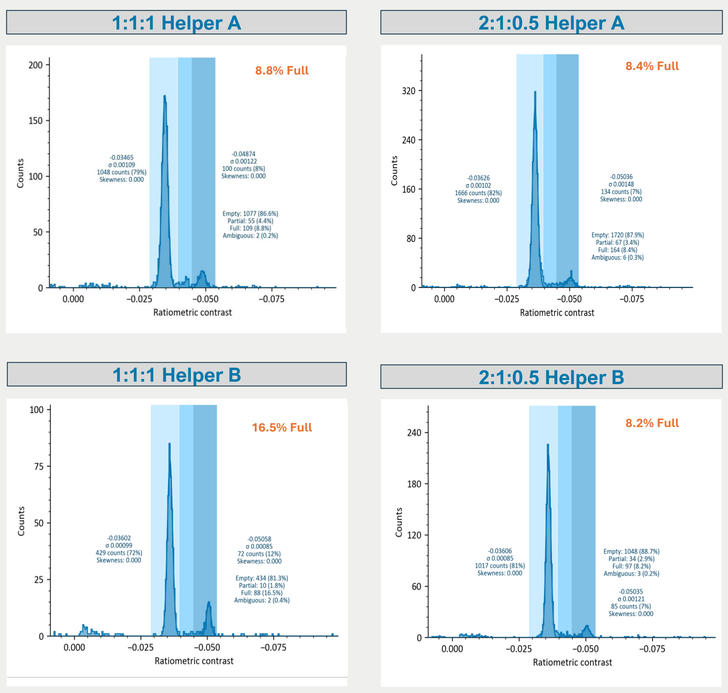


Figure 1: Mass histograms of four clarified harvest samples from cells transfected with different pHelper and two different plasmid stochiometries. The % full particles is highlighted in orange.

The mass histograms (Figure 1) showed that cells transfected with pHelper B at a 1:1:1 ratio produced the highest percentage of full rAAV particles (16.5%) compared to 8.2% with the 2:1:0.5 ratio. Although pHelper A resulted in higher overall productivity (Table 1), the percentage of full capsids averaged 8% for both stoichiometries.

2 MP evaluates DSP parameters

We aimed to develop an efficient purification process for rAAV8, focusing on the separation of empty and full capsids using anion exchange chromatography (AEX). Mass photometry (MP) was employed to monitor the percentage of full capsids and detect potential impurities during the step-gradient elution process.

Empty and full rAAV8 preparations by CsCl gradient ultracentrifugation

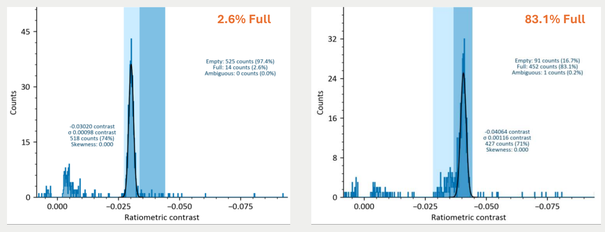


Figure 2: Efficient removal of empty capsids from an rAAV8 preparation measured by MP.

AEX Polishing Purification Study with rAAV8

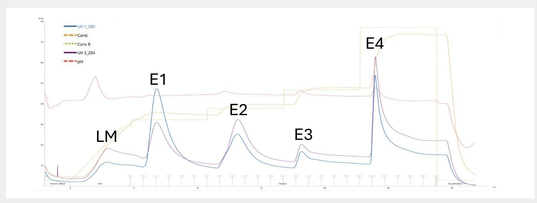


Figure 3: AEX-process chromatogram. Load material (LM) and four elution fractions (E1-E4) were collected and characterized by MP.

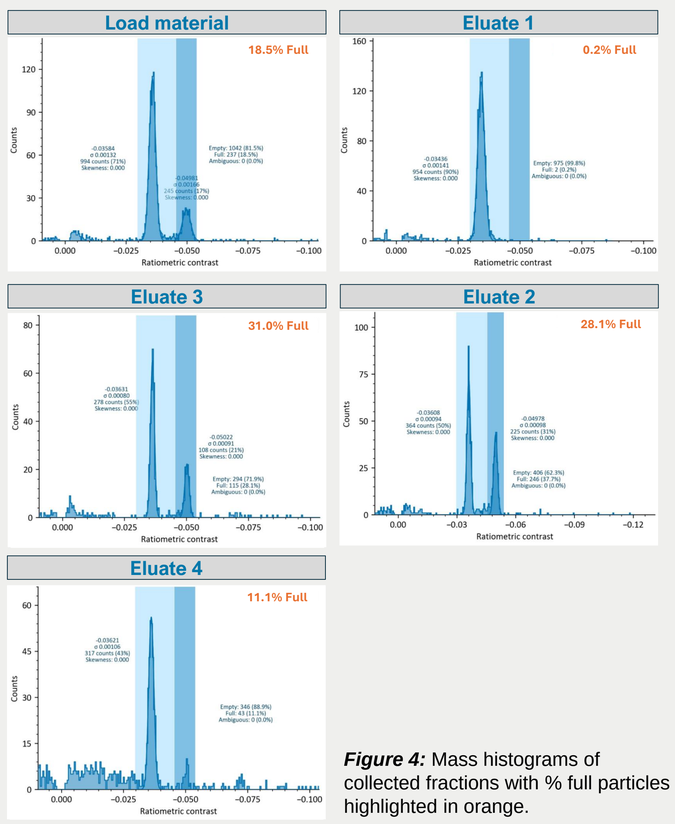


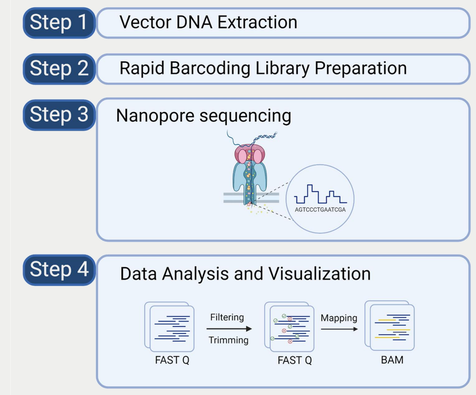
Figure 4: Mass histograms of collected fractions with % full particles highlighted in orange.

Among the elution fractions, E2 and E3 exhibited the highest enrichment of full particles, with 31.0% and 28.1%, respectively. A small population of full capsids (11.1%) was also detected in the final elution (E4), however, a notable proportion of lower-mass impurities was also present.

3 Nanopore Sequencing of Final Products

To investigate thoroughly genome heterogeneity, Nanopore sequencing was employed to analyse the identity of two rAAV preparations. Both contained the same GOI but were produced with different pHelper and pRepCap. NGS was also used to detect other residual nucleic acids.

A.



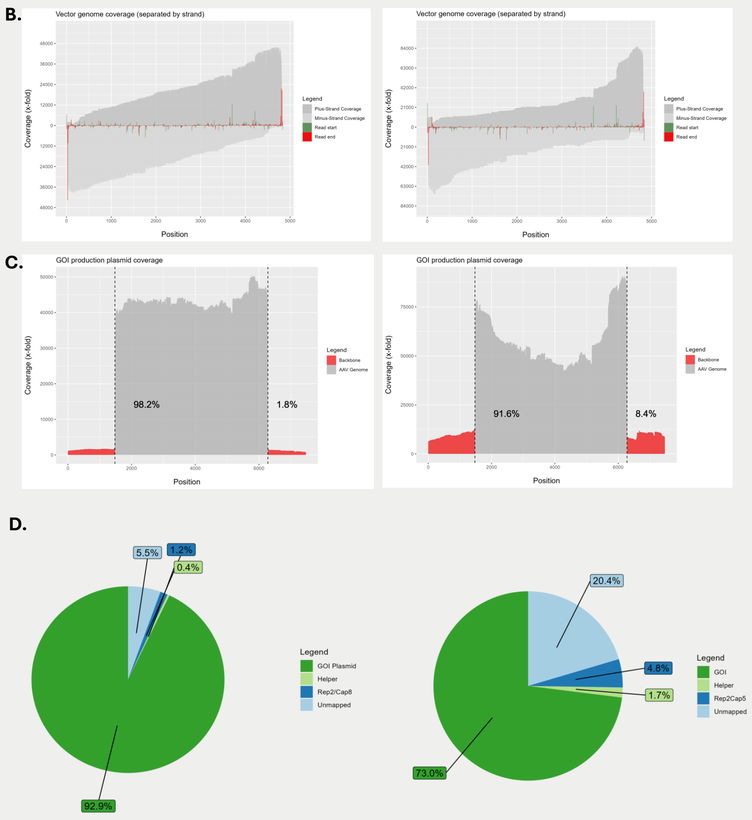


Figure 5: A) Simplified sequencing workflow. B) Coverage analysis of reads aligning to the vector genome from ITR-to-ITR. C) Coverage analysis of reads aligning to the full pGOI. The percentage of mapped reads aligning to the backbone (red) and the vector genome (gray) is displayed. D) Ratios of reads mapped to the respective production plasmids or unmapped reads.

Our in-house bioinformatics pipeline revealed that Vector A showed significantly lower DNA contamination than Vector-B. Specifically, rAAV-A had 1.8% from backbone packaging, 1.2% from pRepCap, 0.4% from pHelper, and 5.5% from host cell DNA, whereas rAAV-B had 8.4%, 4.8%, 1.7%, and 19.7%, respectively.

Conclusions

Our findings underscore the importance of identifying the right analytical tools to understand and optimize each step of rAAV manufacturing and highlight how an agile integration of advanced analytics accelerates process development:

- We effectively used MP to distinguish between empty, partial, and full rAAV particles to identify optimal upstream production parameters.
- MP enabled rapid and accurate monitoring of full-to-empty particle ratios during downstream purification via AEX. This facilitated the selection of the best elution fractions while identifying impurities in other fractions.
- Nanopore sequencing provided detailed insight into genome identity and residual levels from other nucleic acids.

References

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2. Marco T Radukic, et al. Nanopore sequencing of native adeno-associated virus (AAV) single-stranded DNA using a transposase-based rapid protocol, NAR Genomics and Bioinformatics, Volume 2, Issue 4, December 2020, lqaa074, https://doi.org/10.1093/nargab/lqaa074

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