Single-Particle Analysis of AAV Vectors by Mass Photometry: Insights into Packaging Efficiency and Stability

M Wachowius[1], H Martins[1], N Marquardt[1], S Pasupuleti[1], V Ferro[1], R Esposito[1], M Kossek[1], F Leseigneur[1], G Ganjam[1], E Ayuso[1]

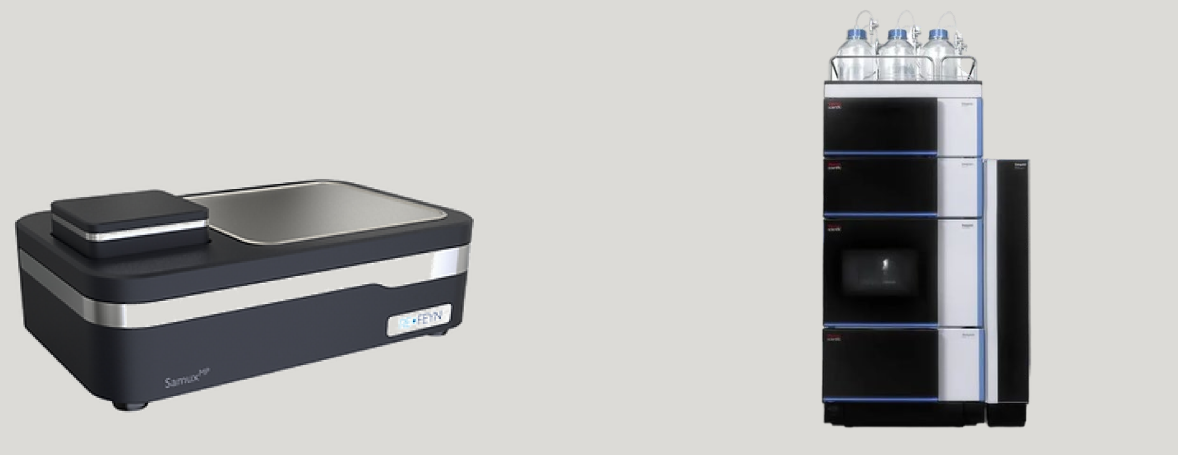
[1] Siegfried DINAMIQS AG, Schlieren, 8952, Switzerland

Contact: **marco.wachowius@dinamiqs.com**

DINAMIQS
a Siegfried company

Introduction

Adeno-associated virus (AAV) vectors are widely used for in vivo gene delivery due to their safety, durable expression, and flexibility. Vector genome **packaging efficiency** is a critical parameter in candidate optimization, influenced by upstream processes and construct design. Incomplete packaging or unwanted encapsidation can reduce yield, potency, and safety. We applied **mass photometry (MP)**, a rapid, label-free, single-particle method, to assess **genome packaging heterogeneity** in AAV. MP resolves **empty**, **partial**, and **full** capsids by **mass differences**[1] with high sensitivity for vector quality assessment. Packaging efficiency in HEK293 suspension cells was significantly improved by 1) comparing **cell lines** optimized for AAV production and 2) by varying plasmid stoichiometry and DNA input. Further, MP was used in tandem with SEC-HPLC to assess **capsid stability**: a highly packaged (>95% full) AAV9 vector exposed to thermal stress showed newly formed empty capsids and low-molecular-mass species, indicating **genome ejection** and capsid degradation. This supports full capsid percentage as a stability-indicating attribute. Overall, MP enables rapid characterization of AAV during upstream development and stability testing, facilitating optimization of packaging efficiency and stability.



Mass Photometry Samux[MP] (Refeyn)

SEC-HPLC Vanquish Flex (Thermo Fisher Scientific)

Results and Discussion

1 Cell line comparison by packaging efficiency

The HEK293-based suspension cell lines A and B were evaluated for AAV production (serotype AAV9) using the same triple transfection, harvest, and clarification protocol. Vector genome titers were determined post-clarification, followed by further purification to enable mass photometry (MP) analysis of particle populations. Cell line A yielded a genome titer of 3.2×10^{11} vg/ml with 62.1% empty, 13.7% partial, and 24.2% full particles by MP. Cell line B produced a comparable titer of 2.2×10^{11} vg/ml but exhibited improved packaging, with 25.7% empty, 29.2% partial, and 45.1% full particles (see Table 1 and Figure 1). These results indicate that while both cell lines achieve similar titers, cell line B provides a substantially higher degree of genome encapsidation while maintaining a viable yield.

Table 1: Overview of the analytical results for the respective cell line.

Condition	% Empty	% Partial	% Full	Yield (vg/ml)
Cell Line A	62.1	13.7	24.2	3.2e+11
Cell Line B	25.7	29.2	45.1	2.2e+11

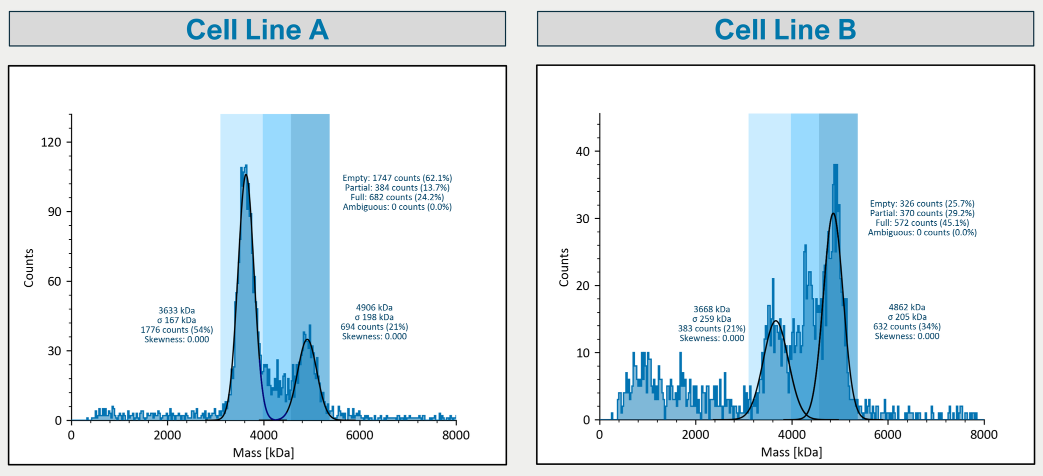


Figure 1: Vector particle mass histograms of samples from cell lines A and B after clarification and further purification to enable mass photometry analysis.

2 DoE plasmid ratio optimization by MP

Upstream conditions for a specific AAV9 construct were optimized to increase the proportion of full particles while maintaining productivity. Suspension cells were transfected using a triple transfection system, with the same cell line applied across all experiments. Six conditions from a full factorial DoE were selected, alongside a platform control. Variables included plasmid ratios and total DNA input. All conditions were processed identically, with vector genome titration performed after clarification and mass photometry (MP) after affinity purification. Genome titers ranged from 9.2×10^8 to 4.9×10^{11} vg/ml, while full particle percentages varied from 6.7% to 39.6%. Condition 2 yielded the highest percentage of full particles with 39.6% with a titer of 1.2×10^{11} vg/ml, whereas Condition 3 achieved the highest productivity (4.9×10^{11} vg/ml) with 21.6% full particles. Together, these conditions define promising points in the design space for balancing yield and packaging efficiency and indicate that those two attributes are to an extent opposing goals.

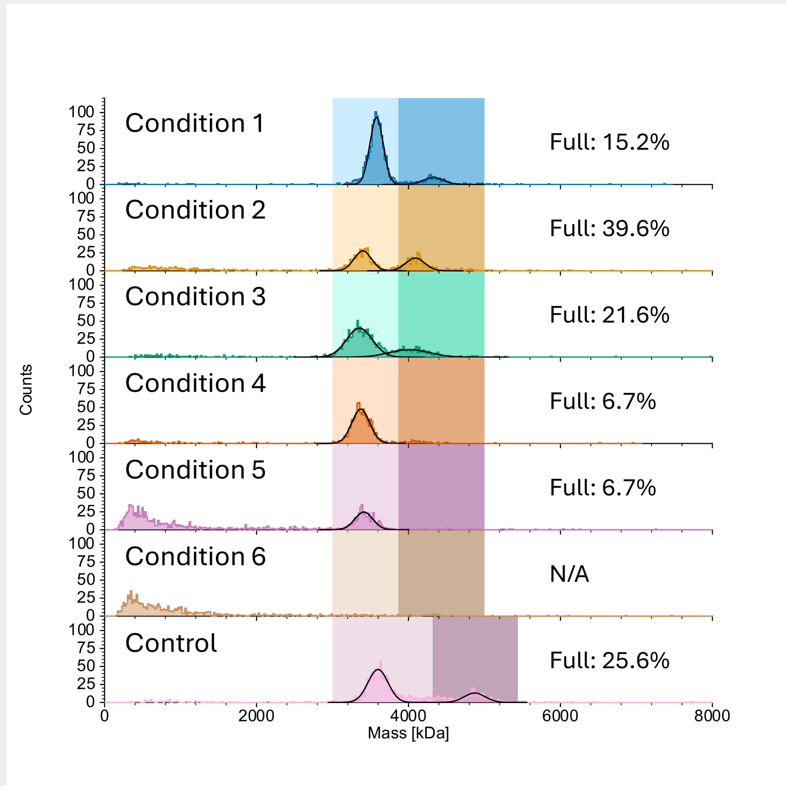


Figure 2: Vector particle mass histograms determined by mass photometry for Conditions 1-6 and an internal platform control.

Table 2: Overview of analytical results for different experimental conditions and control. For condition 6, no clear mass peaks could be obtained in the expected range.

Condition	% Empty	% Full	Yield (vg/ml)
Condition 1	84.8	15.2	3.7e+11
Condition 2	60.4	**39.6**	1.2e+11
Condition 3	78.4	21.6	**4.9e+11**
Condition 4	93.3	6.7	4.1e+09
Condition 5	93.3	6.7	9.2e+08
Condition 6	N/A	N/A	1.0e+10
Platform control	74.4	25.6	4.0e+11

3 Genome ejection under thermal stress

An AAV9 vector highly enriched in full particles (>95%) was subjected to thermal stress to investigate capsid integrity under accelerated stability conditions. Samples were incubated for 5 min at temperatures ranging from 41.8 to 72.3 °C, with untreated vector as control. Capsid stability was assessed by MP to determine single-particle mass distribution, and by SEC-HPLC to monitor aggregation and capsid degradation.

MP revealed no change in mass distribution up to 55.1 °C. At 58.7 °C, lower-mass species appeared with a corresponding decrease in full particles, indicating the onset of particle degradation. From 62.9 °C onward, a distinct population of empty particles emerged, consistent with genome ejection from full capsids, which intensified at higher temperatures, accompanied by an increase in low-molecular-mass species. One distinct 728 kDa species, closely matching the expected mass of the vector genome, was detected, indicating release of genomic DNA from vector particles.

In the same experiment, samples were analyzed with SEC-HPLC as an orthogonal technique to assess the formation of aggregates.

SEC-HPLC confirmed thermal effects, revealing an increase in high-molecular-weight species (0% to ~31% between 41.8 °C and 72.3 °C), indicative of aggregation. The overall chromatographic area decreased with temperature, suggesting formation of very large aggregates not directly assessable by the method.

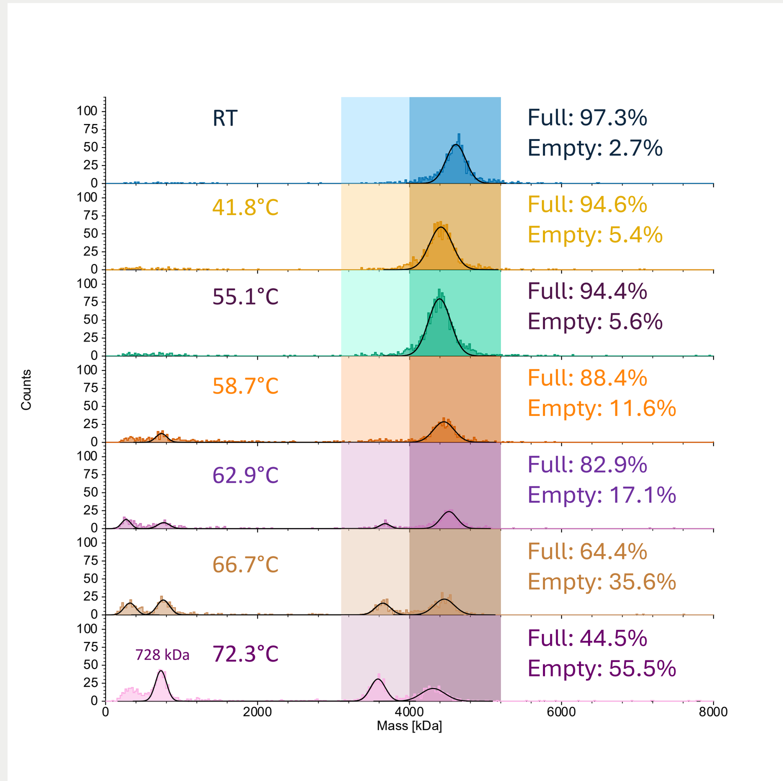
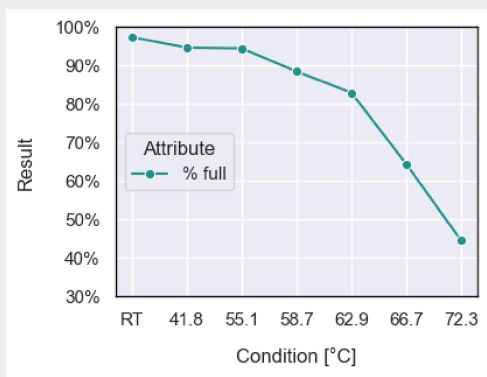


Figure 3: Vector particle mass histograms determined by mass photometry following induction of thermal stress.

Table 3 / Figure 4: Percent full / empty capsids, results and visualization.

Condition	% Full	% Emtpy
RT	97.3%	2.7%
41.8 °C	94.6%	5.4%
55.1 °C	94.4%	5.6%
58.7 °C	88.4%	11.6%
62.9 °C	82.9%	17.1%
66.7 °C	64.4%	35.6%
72.3 °C	44.5%	55.5%



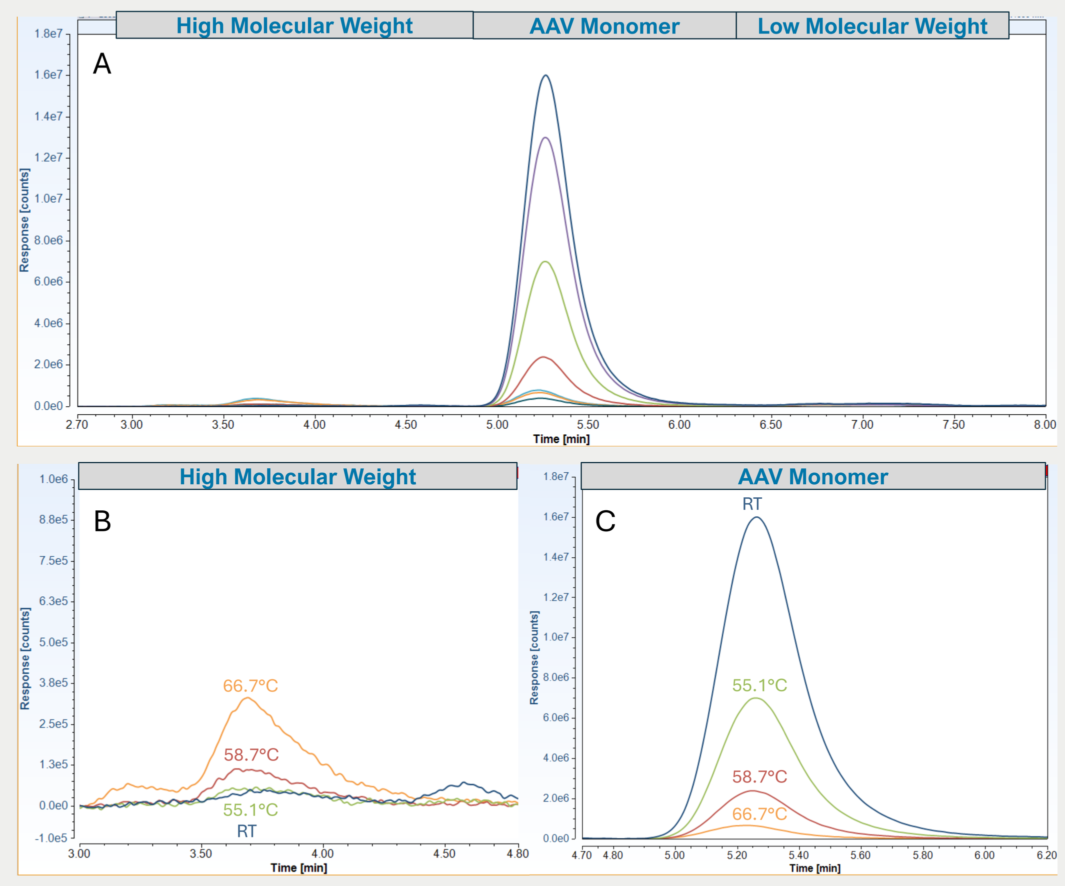


Figure 5: Thermostability of AAV9 assessed by HPLC-SEC. (A) Chromatogram overlay showing the elution profiles across all tested temperatures (41.8-72.3°C). (B) Enlarged view of the high molecular weight region, highlighting a ~30-fold temperature-dependent increase in aggregation (based on peak area). (C) Enlarged view of the monomer peak, illustrating the decrease of intact AAV particles with increasing temperatures. Very large aggregates may not be fully captured by this method.

Together, these results confirm that thermal stress induces genome release and capsid degradation and highlight MP as a direct method to monitor these processes. Accordingly, the proportion of filled particles thus represents both a critical quality attribute and a highly relevant indicator of AAV particle stability.

Conclusions

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

- Mass photometry analysis enabled the optimization of USP conditions, including transfection parameters and cell line selection, to further improve overall process outcomes.
- In tandem with SEC-HPLC, MP was successfully used to investigate the reaction of AAV particles to thermal stress.
- We could observe the formation of AAV particles in the mass range of empty particles, which were most likely derived from genome ejection events. This demonstrates that the percentage of full particles is both a critical quality attribute and a key indicator of AAV particle stability.

References

1. Wagner C., et al., Quantification of Empty, Partially Filled and Full Adeno-Associated Virus Vectors Using Mass Photometry. Int. J. Mol. Sci. 2023, 24, 11033, doi: 10.3390/ijms241311033.
2. Ke Ma, et al., Size-exclusion chromatography of adeno-associated viruses with the SurePac Bio 550 SEC MDi column. Thermo Fisher Scientific, Application note 003089.

Acknowledgements

1. This work is funded by the European Union. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or HaDEA. Neither the European Union nor the granting authority can be held responsible for them. **2.** This work is funded by the UK Research and Innovation (UKRI) under the UK government's Horizon Europe funding guarantee grant numbers 10080927 (The Francis Crick Institute Limited), 10079726 (King's College London), 10082354 (Muscular Dystrophy Group of Great Britain and Northern Ireland) and 10078461 (University College London). **3.** This work has received funding from the Swiss state Secretariat for Education, Research and Innovation (SERI).

Project funded by
Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra
Swiss Confederation
Federal Department of Economic Affairs, Education and Research EAER
State Secretariat for Education, Research and Innovation SERI

MAGIC

Funded by the European Union