Simplifying Progress

High Throughput Screening (HTS) or Single-Column Approach for Improvement of Empty/Full Separation of AAV

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1. Introduction

Developing a reproducible purification process for adeno-associated viral vectors (AAV) is crucial for success during the investigational phase of new drug development and throughout the entire product lifecycle. A key aspect of the AAV purification process is optimizing the polishing step, which aims to separate empty, full, and other subpopulations of AAV. The primary goal of the polishing purification step is to create a process that ensures high recovery while achieving significant enrichment of the potent AAV species. In this study, we present three different approaches using CIM® monolith technology for initial screening of conditions for full enrichment:

- High Throughput Screening with 96-Well Plates: This approach allows parallel processing of up to 96 samples, significantly accelerating the development timeline and providing valuable data for scaling up to larger volumes. The plates can be operated manually using a vacuum manifold, positive pressure manifold, centrifuge, or with a fully automated robotic system.
- CIM® Octa columns: These miniaturized eight-in-line columns are designed for automatic and parallel chromatographic screening of process development parameters. They are operated with a fully automated robotic liquid handling workstation.
- Single Column: This widely accepted method involves screening with a single column, providing a reliable approach to process development.

2. Study objective and experimental design

In this study, we aimed to achieve two main goals:

1. Optimization of Full Enrichment Method for AAV8: We focused on evaluating two key parameters that significantly influence chromatographic full enrichment: pH and elution agent. Our objective was to define the precise elution salt concentration and maximize the yield and purity of the final fraction.

2. Comparison of Screening Approaches: We utilized three different CIM monolithic devices—96-well plates, CIM Octa columns, and single column screening (Figure 1) — to verify that the results are comparable and fall within acceptable ranges.

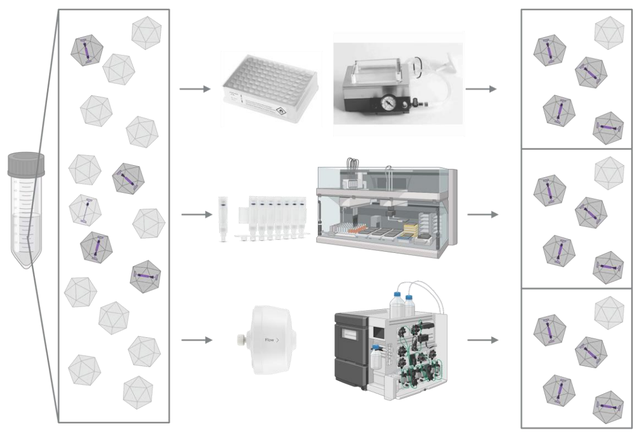

Figure 1: Schematic representation of experimental design.
The experimental evaluation was conducted on the AAV8 serotype with a GFP insert, produced using the HEK293 cell line. The isolation process followed a previously published protocol, which included tangential flow filtration (TFF) and nuclease treatment, followed by cation-exchange chromatography isolation. Sample was than buffer exchanged to loading buffer, and AAV empty and full separation screening was conducted using the CIM QA HR 96-Well Plate (Sartorius, Cat # BIA-120.5213-2), CIM Octa QA HR (Sartorius, Cat # BIA-128.5213-2) and CIMmultus® QA HR 1 mL column (Sartorius, Cat # BIA-311.5213-2). Methods were run using vacuum manifold (Pall), automated robotic system Tecan Freedom EVO 150 base unit (Tecan Group) and Akta Pure 25 (Cytiva).

3. Screening using CIM® 96 Well Plates

The screening experiment using a 96-well plate (Table 1) revealed an increase in tryptophan fluorescence (FLD) in eluate samples at 15 mM for $MgAc_2$ and 20 mM for $MgCl_2$ salt eluent. Higher pH levels enhanced AAV binding but posed risks of capsid damage (data not shown). While FLD data provided insights into overall AAV elution, it lacked details on the separation of full and empty AAV capsids. To address this, samples were transferred to the PATfix® system for additional analysis. The results demonstrated that the $MgAc_2$ eluent achieved superior selectivity and resolution for step gradients, showing significant concentration differences between empty and full capsids compared to $MgCl_2$. The selected conditions involved using $MgAc_2$ as the elution salt at pH 8.5, where empty AAV elution was achieved at 25 mM salt and full AAV at 35 mM (Figure 2).

		Concentration [mM]												
		1	2	3	4	5	6	7	8	9	10	11	12	
pH	**7.5**	A	0	5	10	15	20	25	30	35	40	45	50	100	**$MgAc_2$ eluent**
	8.0	B	0	5	10	15	20	25	30	35	40	45	50	100	
	8.5	C	0	5	10	15	20	25	30	35	40	45	50	100	
	9.0	D	0	5	10	15	20	25	30	35	40	45	50	100	
	7.5	E	0	2.5	5	7.5	10	12.5	15	20	30	40	50	100	**$MgCl_2$ eluent**
	8.0	F	0	2.5	5	7.5	10	12.5	15	20	30	40	50	100	
	8.5	G	0	2.5	5	7.5	10	12.5	15	20	30	40	50	100	
	9.0	H	0	2.5	5	7.5	10	12.5	15	20	30	40	50	100	

Table 1: Experimental Design for 96-Well plate screening. The experimental design for high-throughput screening aimed to identify the optimal elution buffer conditions. Various parameter combinations were tested, including different eluents ($MgAc_2$ and $MgCl_2$), pH values, and eluent concentrations.

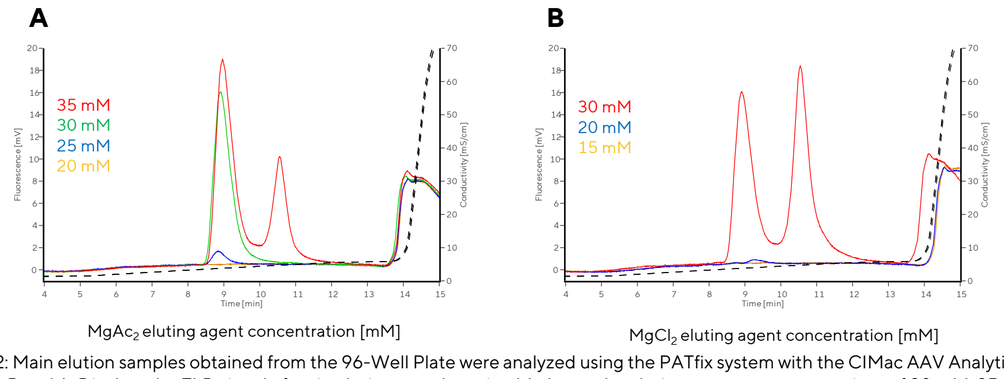

Figure 2: Main elution samples obtained from the 96-Well Plate were analyzed using the PATfix system with the CIMac AAV Analytical column. Panel A: Displays the FLD signal of main elution samples using $MgAc_2$ as the eluting agent at concentrations of 20 mM, 25 mM, 30 mM, and 35 mM, at pH 8.5.
Panel B: Shows the FLD signal of main elution samples using $MgCl_2$ as the eluting agent at concentrations of 15 mM, 20 mM, and 30 mM, at pH 8.5.

4. Screening using CIM Octa columns

The screening on Octa columns involved testing buffers at various pH values to enhance resolution and purity. To effectively monitor both fluorescence and absorbance, the amount of material loaded was 5E+13 vp/ml of monolith. Based on UV absorbance (Figure 3) and PATfix analysis (data not shown), the optimal conditions for full AAV8 enrichment were identified at pH 8.5, similarly as with previous experiment with 96-well plate. The concentration of the elution salt was measured at 27.3 mM for empty capsids and 37.1 mM for full capsids.

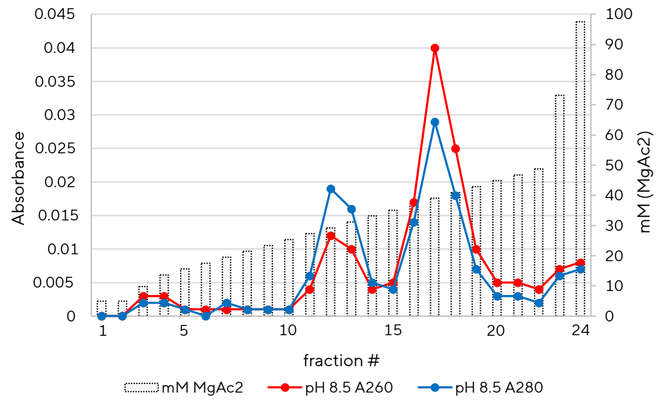

Figure 3: Analysis of Elution Samples from CIM Octa QA HR. Elution samples were analyzed using an absorbance-based microplate reader (BioTek Synergy H1 Hybrid Reader). The absorbance at 260 nm and 280 nm was recorded for all elution samples using $MgAc_2$ as the eluting agent at various pH levels (analysis of fractions from pH 8.5 experiment). Buffer A: 20 mM Tris, 1 % sorbitol, 2.5 % EtOH, 0.1 % Poloxamer 188, pH 8.5 (tested also pH 7.5, 8.0 and 9.0). Buffer B: 20 mM Tris, 50 mM $MgAc_2$, 1 % sorbitol, 2.5 % EtOH, 0.1 % Poloxamer 188, pH 8.5 (tested also pH 7.5, 8.0 and 9.0).

5. Screening using single chromatographic column

In the single column experiment, segmented step gradients were performed, with increments of 2.7 mM salt concentration (6 %) of buffer B (Figure 4). Using a UV detector on the FPLC system we verified that the elution of empty capsids started at 25.7 mM $MgAc_2$, while the elution of full capsids occurred at 36.5 mM salt. Final full AAV fraction was analyzed with various techniques (Table 2).

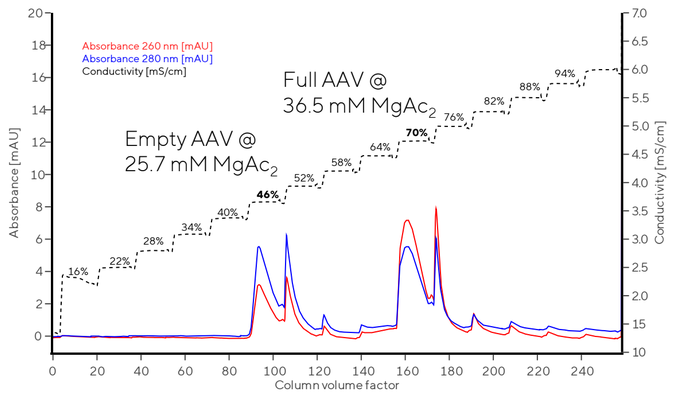

Figure 4: Chromatogram of Elution Step Using $MgAc_2$. The gradients (% of eluent) were adjusted such that the concentration of the $MgAc_2$ eluent was increased by 2.7 mM (6 %) at each step. Buffer A: 20 mM Tris, 5 mM $MgAc_2$, 1 % sorbitol, 2.5 % EtOH, 0.1 % Poloxamer 188, pH 8.5. Buffer B: 20 mM Tris, 50 mM $MgAc_2$, 1 % sorbitol, 2.5 % EtOH, 0.1 % Poloxamer 188, pH 8.5.

Parameter	Result (analytical method)
Recovery	**77 %** (dPCR for vector genome) **79 %** (PATfix AAV platform) **80 %** (Mass photometer)
Purity (% full)	**85 %** (PATfix ultracentrifuge) **99 %** (PATfix + CIMac AAV) **80 %** (Mass photometer) **88 %** (CD-MS)

Table 2: Analytical results for full AAV fraction from single column experiment confirmed recovery around 80 % and purity between 80 and 99 %, with a good alignment between analytical techniques.

6. Conclusion

The results obtained from all three approaches were compared, demonstrating consistency in the elution conductivities of both the empty AAV peak and the full AAV peak, regardless of the method chosen.

- Superior resolution between empty and full AAV8 capsids was achieved with magnesium acetate elution salt at pH 8.5.
- CIM QA HR line provides high reproducibility across different product families and purification scales (Table 3).
- The selection of an appropriate CIM QA HR monolith product for screening is guided by specific laboratory needs and available equipment (Table 4).

	CIM 96-Well Plate			CIM Octa			CIMmultus®
Monolith volume [mL]:	0.2			0.05			1
Loading [vp/mL]:	2.1E+12*			5.2E+13			4.2E+13
Sample consumption / tested condition [vp]:	5.0E+12*			2.6E+12			4.2E+13
Recorded signal:	BioTek Abs	BioTek FLD	PATfix FLD	BioTek Abs	BioTek FLD	PATfix FLD	Unicorn Abs
Start of empty AAV elution [mM]:	below LOQ	30.0	25.0	27.3	27.3	27.3	25.7
Start of full AAV elution [mM]:	below LOQ	n.d.	35.0	37.1	37.1	37.1	36.5

Table 3: Comparison of $MgAc_2$ Concentration for AAV Elution. *Loading amount could be higher to gain possibility of absorbance measurement in elution.

	Type of equipment	CIM 96-Well Plate	CIM Octa	CIMmultus
Lab equipment for screening:	Vacuum manifold	✓	×	×
	Centrifuge	✓	×	×
	Robotic liquid handling workstation (e.g.,Tecan Freedom EVO 150)	✓	✓	×
	Chromatography system (e.g., AKTA Pure 25)	×	×	✓
Direct detection method:	Multi-wavelength ultraviolet (UV) detector, conductivity monitor, pH electrode	×	×	✓
Analytical method:	Microplate reader	✓	✓	✓
	PATfix biochromatography system	✓	✓	✓

Table 4: Selection of QA HR Monolith for Initial Screening. The type of QA HR monolith selected for initial screening is dependent on the available laboratory equipment.