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Method Guide

AAV Full Capsids Enrichment With CIMmultus® QA HR

CIM Convective Interaction Media® chromatographic monolith devices are available in different formats, from high throughput 96-well and 24-well plates, analytical chromatographic columns, and scalable purification columns. The equipment used for different formats (high throughput, analytical, and preparative devices) offers varying capabilities, such as ability to run gradient elution. The following instructions can be directly applied to CIMmultus® preparative columns and CIM® Specimen. Follow format-specific requirements when transferring the conditions outlined here to CIMac™ or CIM® Plate formats.

QA (quaternary amine) is a strong anion exchange ligand. CIM monolith QA HR products provide reproducible purity allowing for enrichment of any full AAV capsids and its chimeras, or surface modified capsids, regardless of the batch or size of the column used. Its main applications are enrichment of AAV full particles. Empty and full AAV capsids have small difference in pI, which can be used for separation using its charge difference for elution. After samples are bound to the column at high pH and low conductivity, they are eluted with increasing linear salt gradient. Empty and full capsids elute at different conductivities due to their charge differences. Example of separation of empty from full AAV particles using QA method, explained in this Method guide, is shown in the Figure 1.

Before CIMmultus QA HR was launched, standard CIMmultus QA was used for this purpose. However, for reproducible AAV empty-full elution profile across the scales we recommend using CIMmultus QA HR instead of standard CIMmultus QA. CIMmultus QA HR is particularly recommended when the goal is to transfer the process from a linear ascending salt gradient to elution in step washes.

For more information, contact
help.bia@sartorius.com

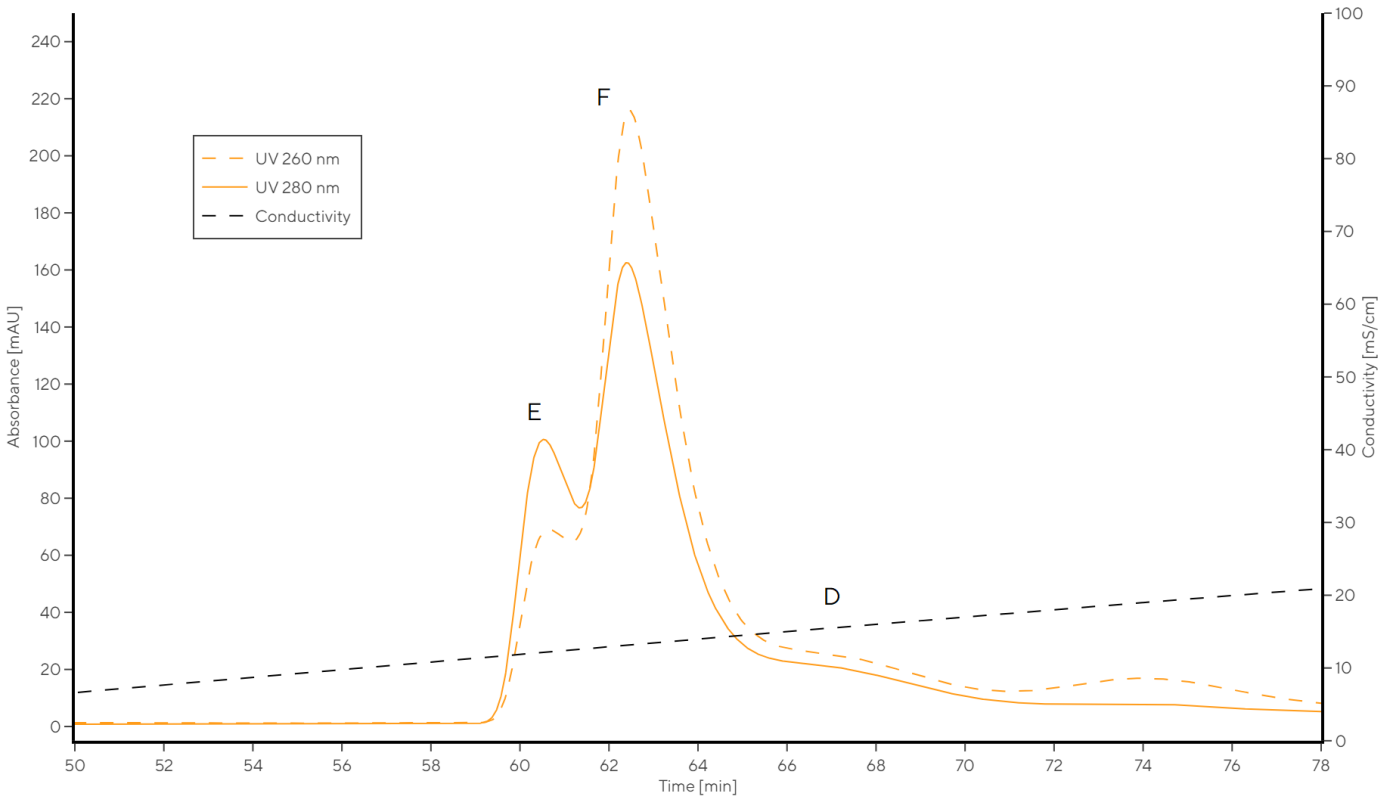

Figure 1: *Typical separation of AAV8 capsids on CIMmultus QA HR (CIMmultus SO3 eluate was applied) using standard QA method: E: empty, F: full and D: damaged.*

AAV is applied on CIMmultus QA HR column at low ionic strength and alkaline pH. AAV have pI at ~ 5,9 - 6,3 and are negatively charged at higher pH values. On CIMmultus QA HR, AAV sample is applied at pH 8.5-9.5. Negatively charged species (including AAV) bind to positively charged QA ligand. Elution is typically achieved with increasing salt concentration or alternatively with decreasing pH gradient. As DNA-based impurities and AAV particles are both negatively charged, they will compete for the binding sites. Better overall purity will be achieved, if sample loaded onto the column is pre-purified: To ensure the most effective enrichment process, it is crucial that the sample attains a high level of purity before being loaded onto CIMmultus QA HR.

Before polishing with CIMmultus QA HR, capture step with either cation exchange interaction chromatography using CIMmultus SO3 or with affinity chromatography is recommended to remove majority of impurities, which could co-elute with full AAV capsids in polishing step and reduce its purity. Contact Sartorius BIA Separations for more information about any of these approaches.

Note: when better separation between empty, partially filled and full AAV particles is needed, we recomend novel QA method, which is patent pending but already available as Cornerstone process development service. For more information visit this link or reach out to help@biaseparations.com.

Getting started

Your column Instructions for Use document can be downloaded by scanning the QR code on the right or by following this hyperlink. CIMmultus columns use a radial flow distribution (Figure 2) inside the housing, requiring the column to be connected to the chromatograph with the correct flow direction. This is different from CIM Specimen that utilize Axial flow through the monolith and therefore when using CIM Specimen flow direction is not important. Note that some chromatographs have default reverse-flow functions built into their software that can cause the flow direction to be reversed without warning. Make sure this function is disabled before conducting any experiments.

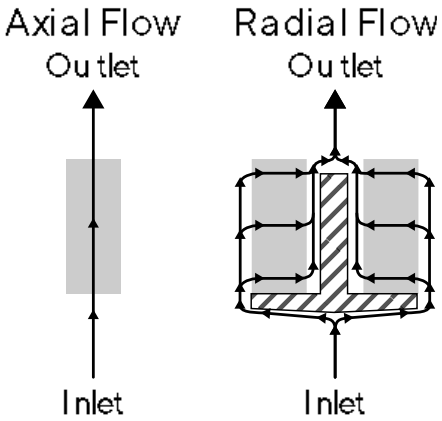

Figure 2*: Comparison of axial flow and radial flow distribution within stationary phase of the chromatographic column.*

Before applying any sample, prepare the column by removing the storage solution as directed in the Instructions for Use document.

Column variants. Quaternary amine (QA) ligand is available as CIMmultus QA or CIMmultus QA HR. For empty full AAV separation, use of CIMmultus QA HR is recommended for high reproducible results.

Sample and preparation. CIMmultus QA HR can be used to enrich AAV capsids containing gene of interest. After capture step, sample should be diluted, or buffer exchanged into binding conditions. If incompatible additives are present in capture step eluate (buffers with relatively strong negative charge properties such as citric acid), buffer exchange is preferred. Sample pH should be basic (between pH 8.5 and pH 9.5) and contain very low concentration of salt for binding (less than 5mS/cm, also depends on the serotype). In buffers, addition of 0.1 % poloxamer, 1 % saccharose and/or 0-5mM $MgCl_2$ can be evaluated.

Different serotypes. Each AAV sample behaves differently in purification steps. Main sources of variability are purity of capture step eluate and serotype of the vector. See below for a few suggestions.

Table 1: *Serotype specific suggestions*

Serotype	Suggestion
AAV2	Addition of 35-50 mM salt is advised to add in Buffer A. This mitigates aggregation.
AAV9	Recommended pH values of running buffers and loading sample: pH 9.2 - pH 9.5. Conductivity of loading sample: below 2 mS/cm for the initial experiment.

Enrichment of full AAV capsids with CIMmultus QA HR

See the optimization and trouble-shooting section for further guidelines.

Buffer A. Binding buffer | Wash buffer | Dilution buffer. 25 mM BTP, 0.1 % Poloxamer, 1 % sucrose, pH 9

Buffer B. Elution buffer. 25 mM BTP, 0.5 M KCl, 0.1 % Poloxamer, 1 % sucrose pH 9

Buffer C. Deionized water.

Cleaning buffer. Refer to Table 2 or to the corresponding Instructions for Use document.

Column neutralisation buffer. Refer to Table 2 or to the corresponding Instructions for Use document.

Sample neutralisation buffer. 1M BTP, pH 6.5

Sample dilution buffer. Buffer A

Flow rate. Start with a flow rate of 5 CV/min on CIMmultus 1 mL. It is recommended to maintain the same flow rate in CV/min during scale-up.

Column preparation. Please refer to Instructions for Use document before first use, integrity test should be performed to check if monoliths integrity. Before each use, storage solution should be washed out and column equilibrated with elution and binding buffer. If using cGMP environment, columns should be sanitized and neutralised prior equilibration according to the Instructions for Use document.

Column equilibration. If needed, wash the column with at least 10 CV of water to remove storage solution. Equilibrate the column with at least 10 CV of elution buffer (buffer B), and then pump at least 10 CV of binding buffer through the column or until conductivity and pH at column outlet match conductivity and pH at column inlet.

Load sample. Dilute the AAV sample in sample dilution buffer (buffer A) until conductivity of sample is below 5mS/cm and load it on the column. In some cases, buffer exchange is preferred instead of dilution (please see Optimization and Troubleshooting section). Observe operating pressure during application of large volume samples. Reduce flow rate if necessary to maintain operating pressure within acceptable limits.

Wash with buffer A. Wash to return UV signal to baseline.

Elute with buffer B (linear gradient). Elute AAV with linear gradient from 0 % Buffer B to 50 % Buffer B over 50 CV (forming the gradient with Buffer A). After linear gradient, perform strip with step gradient to 100 % Buffer B and hold it for 5 CV. Collect the fractions of elution peaks. For optimal fraction collection, collect next fraction when UV 260 nm cross UV 280 nm line. Collect next fraction, when UV line drops close to the baseline or when it starts to increase again. Add sample neutralization buffer to the collection tubes to ensure proper pH adjustment of the eluted samples to pH 8.

Wash with buffer C. If needed, wash the column with at least 10 CV of water to prevent mixing of incompatible buffers (NaOH and $MgCl_2$).

Column cleaning. Treatment with cleaning buffer is recommended after every run. The contents of the cleaning step may be collected and neutralised/buffer exchanged for further analysis.

Cleaning procedure of CIMmultus QA HR and CIMmultus QA are summarized in the table below. For more information on each recommended cleaning procedure, please refer to Instructions for Use.

Table 2: *CIMmultus® QA HR and CIMmultus® QA cleaning procedures*

	QA HR	**QA**
Cleaning solution	0.1 M NaOH + 2 M NaCl	1 M NaOH + 2 M NaCl
Column neutralisation buffer	0.5 M phosphate buffer pH 6.5 or 1 M sodium acetate pH 5.5 or 1 M ammonium acetate	100 mM Tris + 1 M NaCl pH 7 or 1 M ammonium acetate
Storage buffer	20 % Ethanol in 5 mM sodium phosphate at pH 6.0	20 % Ethanol
Cleaning procedure	If needed wash the column with 10 CV of water to prevent mixing of incompatible buffers (e.g. $MgCl_2$ and NaOH)	
	Wash the column with at least 10 CV of cleaning solution	
	If needed wash the column with 10 CV of water to prevent mixing of incompatible buffers	
	Wash the column with at least 10 CV of a column neutralisation buffer.	

Storage. After completed cleaning procedure (including adequate neutralization step), wash the column with 10 CV of water and then pump at least 10 CV of storage solution through the column. For appropriate storage solution refer to Table 2 or Instructions for use document. Take special precautions to avoid following NaOH directly with ethanol as this will form ethoxide radicals that may significantly degrade the ligand in minutes. The column must be at near-neutral pH before introducing ethanol.

Optimization and Troubleshooting

Use the initial scouting chromatogram as a guide for optimizing the composition and duration of the individual steps described above.

Main points of optimisation:

- Salt: screen chlorides and acetates with various cations (Na, K, Mg, etc.)

- pH: 8.5 - 9.5
- Loading amount (starting point: 5E+12 - 1E+13 vp/mL)
- Buffer concentration and buffer system: Tris, BTP
- Additives: magnesium, poloxamer, sugar
- Sample preparation: dilution, buffer exchange
- Filtration step
- Gradient slope | duration

Effects of pH. CIMmultus QA HR is strong anion exchanger, which retain charge throughout whole pH range. Process is run at alkaline pH. At an elevated pH level, the binding strength of AAV increases, but at the same time, introduces a potential risk of capsid damage. For AAV9, suggested pH for evaluation is pH 9.2 - 9.5. For other serotypes, pH 9 is usually sufficient.

Effects of salts. Optimization of salt concentration for loading and washing may be required for each AAV sample to achieve optimal binding to the column and selective elution. Typically, it is advisable to perform initial process development run with the lowest possible salt concentration for loading, as certain samples could elute at very low salt concentrations. If sample is prone to aggregation (e. g. AAV2 serotypes), addition of salt in the loading sample might be tested to prevent aggregation. The most common eluent is KCl. Other salts could also be investigated, such as NaCl, K-acetate or Na-acetate.

Effects of other additives. Additives in buffers and/or in sample will affect separation, purity and/or recovery. For effect of different additives see table 3 below.

Table 3: Effect of different additives

Additive	Effect
Magnesium ions	$MgCl_2$ is common additive in empty and full AAV process, as it stabilizes empty capsids and may change elution profile. Suggested concentrations for testing are in range 0 mM to 5 mM.
Poloxamer	Poloxamer in concentrations between 0.01 % and 0.2 % can be added to the buffers to prevent non-specific binding to laboratory equipment (filters, tubing, glassware,...). With its addition recovery of the sample is promoted.
Sugar	With addition of sugars (sucrose or sorbitol), stability of AAV capsids is increased, as sugar increases osmolarity of the sample and prevents aggregation. At the same time conductivity is not increased and sample can easily bind to the column without aggregation effects due to low conductivity. 1% of the sugar can be added in sample and buffers.
Citric ions	Citric ions are negatively charged metal-chelating agent and bind to AEX columns. They compete with negatively charged sample for the binding sites. Citric ions chelate with magnesium ions and thus negate Mg role in buffers. If using citric acid as an affinity elution agent in a step elution approach, it is often difficult to control the amount of citric acid in the loading sample for the next chromatography step. If the amount of citric acid is not fully controlled (i.e. exactly the same fractionation approach regardless of purification system, sample volume or operator), the varying amount of citric acid can be expected to be a source of variability (i.e. non-reproducible QA elution profile). Change in chromatographic performance might be seen even at low concentrations or low amounts of citric ions present in sample. If citric ions are present in affinity eluate, buffer exchange is highly recommended for more robust polishing step with this method. Alternatively, eluent in affinity step can be changed to some other (e.g. formate).

Effects of insufficient column equilibration. Column equilibration has a significant impact on chromatography performance. Without efficient equilibration, sample may not efficiently bind to the ligand, resolution between empty and full could worsen etc. To efficiently equilibrate the column, at least 10 CV of solution for each step should be used. To charge the column with suitable counter ion, salt concentration higher than those in elution buffer might be needed.

Cleaning. Inadequate cleaning may be indicated by a gradual increase of operating pressure over a series of runs, increased tailing during the post-load washing step, a change in the shape of the elution peak, increased contamination of the eluted sample, and reduced recovery.

Effects of loading amount. Dynamic binding capacity of very pure AAV sample can be greater than 1E+15 vp/mL monolith. Depending on the amount of impurities present in the sample, DBC is usually lower. Ratio of empty and full capsids will also affect binding capacity. If there are more empties in the original sample, loading amount in "vg/mL" to achieve desired purity will be lower.

However, we would not recommend loading more than 10-15 % of DBC because loading more worsens the empty-full resolution and starts elution earlier in the gradient. With higher amount of loading AAV, elution of peaks shifts to the left (it starts lower in the gradient), and resolution is worsened.

Therefore, rather than testing breakthrough, we recommend testing different loading amounts to achieve optimal purity (separation). Different amounts are loaded (e.g., 1E+12, 5E+12, 1E+13, 5E+13, 1E+14 vp per ml of stationary phase) and changes in resolution, peak shape, etc. observed to determine the concentration at which the elution profile remains optimal (e.g., resolution is maintained, empty particles start eluting at the expected conductivity).

With sufficient capture step, 1E+14 vp/ml monolith is a workable optimal range still providing consistent peak shape and elution of AAV at the expected conductivity values (for all serotypes).

Further reading

- Specimen QA HR Method Guide – on request
- Roadmap to success in AAV purification. In-process control, high throughput & novel column modalities as necessary means for control over scalable AAV process (https://www.biaseparations.com/en/library/publications/1185)

FAQ

Does sample need to be purified prior polishing step?

Yes. Our recommendation is performing capture step on CIMmultus SO3 column (strong cation exchange column). Before the capture step, pre-treatment is also important to further reduce impurities and to increase the binding capacity on SO3 column. Suggested methods for pre-treatment are (alone or combined): tangential flow filtration | DNAse treatment | CIMmultus OH chromatography | flocculation | solid phase extraction.

What is the typical dynamic binding capacity of CIMmultus QA HR column?

Look above in Optimization and Troubleshooting chapter, Effects of loading amount.

What operating flow rate do you recommend for each column size?

The minimum and maximum flow rate for each column is defined in the Product Sheet, downloadable from: https://www.biaseparations.com/en/certificates.

Flow rate does not affect the resolution and capacity.

How to store the column? Can the column be stored in a different solution than 20 % Ethanol?

Recommended storage solution for QA HR is 20% Ethanol in 5 mM sodium phosphate at pH 6.0.

Recommended storage solution for QA is 20 % Ethanol, but the column can also be stored in working buffers overnight.

More information can be found in the Product Sheet, downloadable from: https://www.biaseparations.com/en/certificates.

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