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Method Guide

Enrichment of Full AAV With CIMmultus® PrimaT

CIM General Instructions for Use (IFU) document can be accessed by scanning the QR code on the right or by following this hyperlink. This method guide is a separate document written specifically for the use of CIMmultus® PrimaT for AAV purification, in particular to guide the user on how to start CIMmultus PrimaT purification for enrichment of full AAV capsids.

CIMmultus PrimaT is a monolithic chromatographic multimodal column mainly used for purification of adeno-associated virus (AAV) to separate AAV sub-species. Separation is based on weak anion exchange, H-bond and metal affinity coordination interactions with the AAV. CIMmultus PrimaT allows binding of all AAV serotypes and provides advanced removal of product-related impurities and AAV sub-species, while maintaining high recovery rates.

Main benefits of CIMmultus PrimaT:

- **AAV binding at neutral or near neutral pH**: Multimodal properties of CIMmultus PrimaT allow binding of AAV at neutral or near-neutral pH values
- **Enables separation of different AAV subpopulations:**. Unique multimodal elution separation mechanism usually leads to separation of full AAV from different product related impurities.
- **Endotoxin Removal:** Allows at least 5 log of endotoxin removal

Due to the multimodal properties of CIMmultus PrimaT column, elution based on different concentrations of metal ions is recommended, such as starting with an ascending linear MgCl2 gradient. Characteristics of binding and elution interactions between AAV and CIMmultus PrimaT vary for each specific AAV capture eluate sample, primarily dependent on the purity of capture step eluate and AAV serotype.

This method guide describes two individual purification methods, specifically tailored to the selected AAV serotype. Method 1 is mainly dependent on weak anion exchange properties and metal affinity coordination effects and Method 2 utilize also H-bond interactions for AAV binding. The main features of both methods are described in Table 1.

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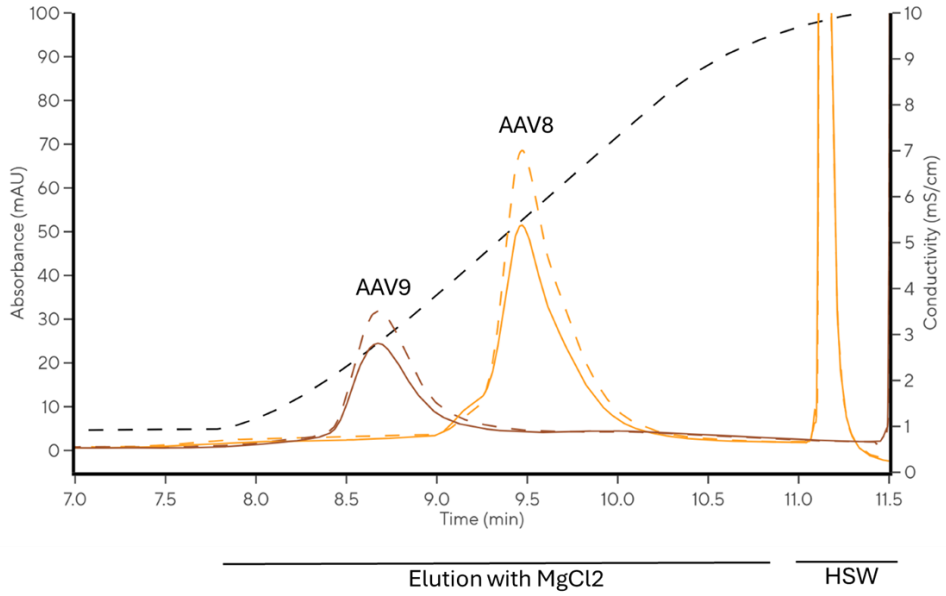
Table 1: *Main features of recommended CIMmultus PrimaT AAV purification methods*

	Method 1	**Method 2**
Serotypes	Versatile method that works with variety of AAV serotypes, tested with AAV2, AAV8, **AAV9** and AAVrh10	**Preferred method for AAV8**. In our experience this method doesn't work with AAV9. Typically separates several AAV subpopulations, besides empty AAV and full AAV
Binding	**Binding at pH 8.5** with addition of Sucrose and Poloxamer	**Binding at pH 7** with addition of Sucrose and Poloxamer
Wash after loading	Wash with binding buffer	Wash with binding buffer and then **wash with buffer at pH 9.0**
Elution	AAV elute in two separate, distinct elution phases: first elution phase with **ascending MgCl2 concentration**, in which empty and full AAV capsids elute. Remaining empty AAV capsids elute in the second elution phase at **high salt wash**	

Both methods were tested on most commonly used AAVs in gene therapy (i.e. AAV2, AAV8, AAV9 etc.). Figure 1 illustrates example elution chromatography profiles AAV8 and AAV9 serotypes using the same method.

Figure 1: *Chromatographic profile of the elution of AAV8 (orange) and AAV9 (brown) using CIMmultus PrimaT column following Method 1 (isocratic pH). CEX-SO3 purified sample AAV8 and AAV9 samples. E/F ratios: AAV8 = 47 %, AAV9 = 31 %. AAV loading amount: AAV8 = 2.7E+13 vp/per mL column, AAV9 = 1.5E+13 vp/per column. Black dashed line represents conductivity values, colored dashed lines represent UV absorbance at 260 nm, colored solid lines represent UV absorbance at 280 nm, high salt wash (HSW).*



Fine tuning of the method is required due to the high variability of AAV samples – different serotypes, post-translational modifications, different lengths of insert and process variabilities (variability of upstream processes and purification conditions prior to polishing).

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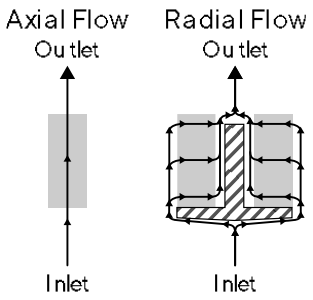
Getting started

In CIMmultus columns, flow is radial, moving from the outer side through the monolith to the inner side (Figure 2), requiring the column to be connected to the chromatograph with the correct flow direction. This is different from CIMac that utilize axial flow through the monolith and therefore when using CIMac 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 with CIMmultus columns.

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



Purification protocol

Sample preparation: After the capture step, samples should be immediately buffer exchanged (BX) to the Buffer A1 to minimize salt concentraces and traces of residuals in capture step buffers to enhance reproducibility of CIMmultus PrimaT purification results.

Column integrity test: It is highly advised to first perform an integrity test to check the monolith's integrity. Instructions are available by scanning the QR code on the right or by following this hyperlink.

Flow rate: Chromatography with monoliths ensure capacity and resolution independent of the flow rate. However, typical starting flow is about 5 CV/min for a CIMmultus 1 mL column. When scaling up, adjust the flow rate according to the IFU for specific CIMmultus column size.

Column preparation: Before each use, storage solution should be washed out and column should be sufficiently cleaned, neutralized and equilibrated, as described in Column equilibration chapter. List of recommended buffers is referred in Table 2.

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Table 2: Buffers for initial purification methods. *Titration to final pH ± 0.05 is needed. ** No titration is needed; mixture of Tris/Boric acid should give a pH value of 9.0 ± 0.05

	Purpose	**Method 1**	**Method 2**
Buffer A1	Binding	10 mM BTP, 1 % saccharose, 0.1 % poloxamer pH 8.5*	25 mM HEPES, 1 % saccharose, 0.1 % poloxamer pH 7.0 *
Buffer A2	pH transition	/	50 mM Tris, 13.6 mM borate, 1 % saccharose, 0.1 % poloxamer pH 9.0 **
Buffer B1	Elution	10 mM BTP, 50 mM MgCl2, 1 % saccharose, 0.1 % poloxamer pH 8.5*	50 mM Tris, 9.6 mM borate, 50 mM MgCl2, 1 % saccharose, 0.1 % poloxamer pH 9.0 **
Buffer B2	Salt strip & Neutralization	10 mM Tris, 2 M NaCl, 1 % saccharose, 0.1 % poloxamer pH 8.5*	
Buffer C	Prevent direct mixing of incompatible buffers	Deionized water	
Cleaning buffer (1M NaOH with 2M $NaCl_2$)	Maintain column performance and restore column capacity	Refer to the corresponding Instructions for Use document.	
Sample neutralisation buffer	Maintain AAV stability and ability to infect cells	/	1M BTP, pH 6.5

Column equilibration:

- **Initial wash:** If column is stored in 20 % Ethanol, begin by washing it with at least 10 CV of water. This step is crucial to prevent mixing of ethanol residues from mixing with the mobile phase buffers.
- **Cleaning and neutralization**: Wash the column with 10 CV cleaning buffer, followed with 5 CV high salt buffer (buffer B2).
- **Prime buffer B line:** Prime buffer B line with Buffer B1.
- **Equilibration:** Equilibrate the column using buffer A1 line with at least 20 CV of binding buffer (buffer A1) or until conductivity and pH at column outlet match conductivity and pH at column inlet.

Load sample: Observe operating pressure and if necessary, reduce flow rate to maintain operating pressure within acceptable limits of the column (as noted in IFU) and within acceptable limits of the FPLC system.

Wash:

- **Method 1:** Wash with 10 CV buffer A1 to return UV signal to baseline.
- **Method 2:** Wash with 10 CV buffer A1 to return UV signal to baseline. Then wash with 10 CV buffer A2 for pH transition to alkaline pH.

Elute with buffer B1 (linear gradient). Elute AAV with linear gradient to 100 % Buffer B1 over 20 CV:

- **Method 1:** Gradient is formed from 100 % buffer A1 to 100 % buffer B1.
- **Method 2:** Gradient is formed from 100 % buffer A2 to 100 % buffer B1.

High salt strip. After linear gradient, perform strip with step gradient to 100 % Buffer B2 and hold it for 10 CV. If needed, add sample neutralization buffer to the collection tubes to ensure proper pH adjustment of the eluted samples to pH 8.

Wash with buffer C. 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. Cleaning fractions may be collected, immediately neutralised/buffer exchanged for further analysis.

For more information on recommended cleaning procedure, please refer to IFU.

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Storage. After completed cleaning procedure (including neutralization step), wash the column with 10 CV of water and then with at least 10 CV of storage solution. Storage solution is noted in the IFU. The column must be at near-neutral pH before introducing ethanol.

Optimization, Troubleshooting and Other Considerations

Optimization of the purification method require tailoring purification process to specific AAV sample due to their high variability. When optimization is desired, consider screening following variables:

- **pH value:** pH value impact anion-exchange and hydrogen bond interactions of CIMmultus PrimaT with AAV. It is recommended to screen different pH values for loading and elution conditions
- **Buffering system:** When optimizing pH value, a different biological buffering system might be chosen to support buffer capacity at that value, but this can influence AAV elution performance.
- **Specific additives to promote or inhibit hydrogen bonding or metal-chelating effect interactions:** Effects of $MgCl_2$ in the load, Sorbitol, PEG, Urea and other possible additives that can effect these interactions.
- **Column capacity:** Defining optimal loading range.

More information on how these variables impact full AAV enrichment using CIMmultus® PrimaT are available in Application note: Exploring Chromatographic Parameters for Enhanced Full AAV Enrichment With CIMmultus® PrimaT

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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) prior to polishing step for AAV empty-full separation. Prior to capture step, pre-treatment is also important to further reduce impurities and to increase the binding capacity on CIMmultus SO3 column. Suggested methods for pre-treatment are (alone or combined): tangential flow filtration | DNAse treatment | CIMmultus OH chromatography | flocculation | solid phase extraction.

What operating flow rate do you recommend for each column size?
The minimum and maximum flow rate for each column is defined in the Instructions for Use document, downloadable from: Product Documentation (https://www.biaseparations.com/library/product-documentation/). 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 PrimaT is 20 % Ethanol. More information can be found in the Instructions for Use, downloadable from: Product Documentation.

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

Germany
Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Stedim North America Inc.
656 Johnson Avenue
Bohemia, NY 11716
Toll-Free +1 800 368 7178

Slovenia
Sartorius BIA Separations
Mirce 21
5270 Ajdovscina
Phone +386 596 995 00

For more information, visit

www.sartorius.com

www.biaseparations.com