Application Note

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CIMmultus SO3, Cation exchanger, AAV capture, chromatographic parameters

Exploring Chromatographic Parameters for AAV Capture Step with CIMmultus® SO3

Abstract

This application note explores the impact of various parameters on the performance of the CIMmultus® SO3 column. CIMmultus SO3 is a strong cation exchanger (CEX) that binds molecules with a predominantly positive charge and repels molecules with a predominantly negative charge across the entire pH range.

CIMmultus SO3 allows binding of all AAV serotypes and provides advanced removal of process-related impurities, such as host cell proteins (HCP) and DNA (hcDNA), while maintaining high recovery rates. Screening different pH values and salt concentrations impacts sample binding and elution profiles, thereby affecting the purity of the AAV eluate. Additionally, sample pretreatment is an important factor, as it influences the column's capacity. The column's scalability was confirmed with AAV loading density experiments.

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Introduction

AAV purification process begins with the collection of supernatant or lysate from the cell culture, followed by a clarification and capture step. The main goal of capture step is efficient removal of process-related impurities (host cell DNA and proteins). CIMmultus SO3 monolithic column offers a versatile solution for AAV capture step, enabling efficient binding and elution of any AAV serotype and engineered AAV capsids.

Similar to other CIMmultus monoliths, CIMmultus SO3 is ready-to-use, scalable, and caustic stable, enabling multicycling purification. Initial protocol for using the CIMmultus SO3 column is described in the following method guide: AAV Capture Step with CIMmultus SO3.

The purpose of this application note is to showcase the effect of different factors influencing performance of the CIMmultus SO3 column for AAV capture step. Given the diversity of AAV capsids, screening various chromatography conditions is often required to achieve optimal purification results with specific AAV serotype or to demonstrate the robustness of the selected purification method. When screening is required, understanding the impact of different process parameters is crucial.

CIMmultus SO3 advantages:

- Binds and elutes any AAV serotype (Figure 1), including engineered capsids
- Reusable column (cleanable with 1M NaOH) (Graph 3)
- High recovery and purity

A side-by-side comparison of CIMmultus SO3 and commercially available affinity resins demonstrated several key advantages of CIMmultus SO3. In parallel experiments, columns were evaluated for process and step recoveries, impurity reduction, product capacity, and processing time. Case studies are presented in the following posters:

Comparability Study Between Ion-Exchange Monolith and Affinity Resin For Purification of AAV8:

- Superior recovery: Achieves 41-42% overall process recovery, compared to 30-31% with the affinity approach, ensuring higher recovery of full AAV capsids.
- Enhanced purity: Offers better removal of host cell proteins and empty capsids compared to affinity ligands, improving subsequent polishing steps.
- Exceptional binding capacity: Shows superior capacity, with a TFF step increasing capacity by 17-fold compared to direct harvest loading. The affinity column's capacity wasn't assessed with clarified harvest due to impractical loading times exceeded 100 hours.

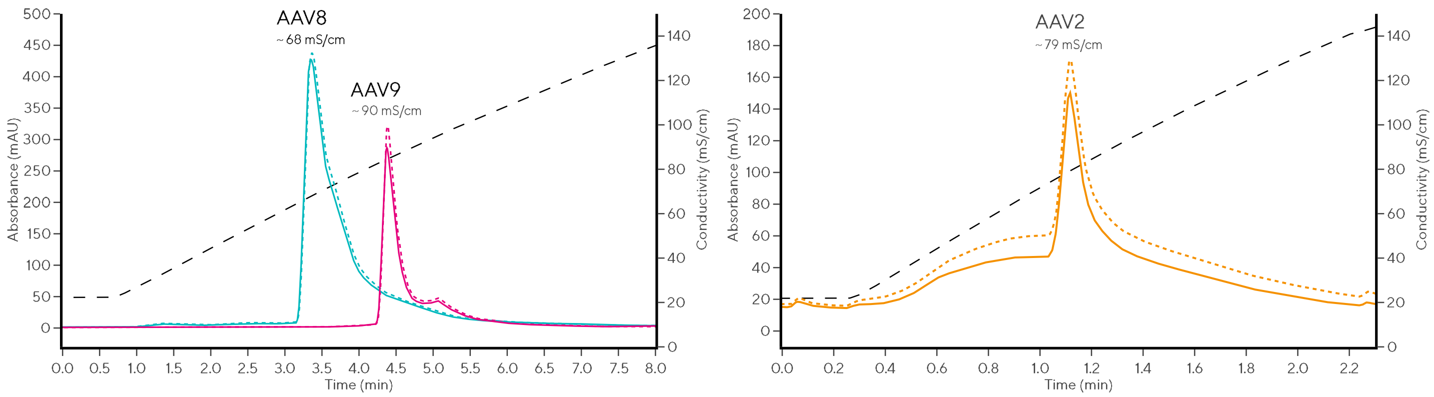
Comparing Cation-Exchange Monolith and Affinity Resin for Chromatographic Capture and Purification of rAAV:

- Rapid processing time: Purification times were 2.6 times faster than the Affinity-Antibody resin-based column and 1.5 times faster than the Affinity-Peptide column.
- Superior recovery: 8% higher step recovery for rAAV9 with CIMmultus SO3 compared to Affinity-Peptide column.

AAV Serotype

CIMmultus SO3 is proven to bind AAV1, AAV2, AAV3, AAV5, AAV6, AAV8, AAV9, AAV10, and AAV11 serotypes. Minor optimization may be required for each specific serotype. Various AAV serotypes were evaluated using CIMmultus SO3 column with a linear gradient elution from 0 to 2 M NaCl at pH 3.5. Different AAV serotypes elute at different conductivity values. For example, AAV8 and AAV2 are expected to elute at lower conductivity values than AAV9 (Figure 1).

Figure 1: *(left) Overlay of AAV8 (teal) and AAV9 (magenta) elution profiles using a CIMmultus SO3 column 80 mL column and (right) AAV2 (orange) elution profile using CIMmultus SO3 8 mL column. Clarified AAV8 and AAV9 harvest samples were pretreated using TFF coupled with salt-tolerant nuclease, followed by acidification and filtration, whereas AAV2 was pretreated using CIMasphere AAV particles and filtered, followed by acidification and filtration. AAV loading amount: AAV8 = 5.2E+13 vp/mL column, AAV9 = 4.0E+13 vp/mL column, and AAV2 = 1.8E+13 vp/mL column. Black dashed line represents conductivity values, colored dashed lines represent UV absorbance at 260 nm, and colored solid lines represent UV absorbance at 280 nm.*



Loading Conditions

Salt Concentration

CIMmultus SO3 binds positively charged particles, such as AAV and host cell proteins. AAV and the majority of host cell proteins are positively charged at low pH values. Optimizing the salt concentration in the AAV loading sample can enhance column capacity by preventing less positively charged host cell proteins from binding to the column. For optimal loading, it's recommended to use the highest salt concentration that still allows efficient AAV binding. A minimum salt concentration of 100 mM is recommended, as exposing AAV to lower concentrations can cause the virus to aggregate. Most serotypes elute around 1 M salt. To determine the optimal salt concentration for AAV binding, it is advisable to gradually increase the salt levels.

pH value

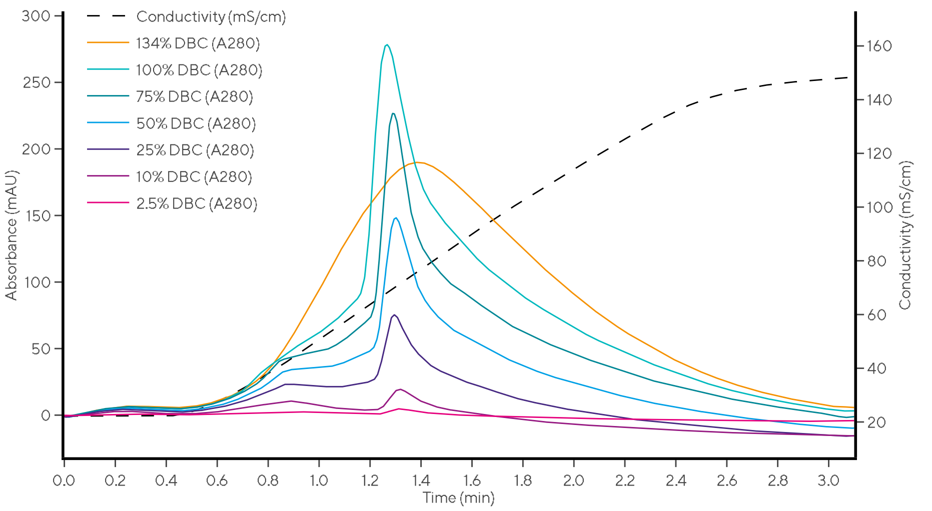
AAV elution is usually performed using a salt gradient, with consistent pH values across the entire purification run. Recommended pH values are in the range from 3.5 to 5. pH values at the upper end of this range (e.g. pH 5.0) are expected to result in higher recovery but may compromise purity. Conversely, lower pH values are expected to yield a more pure elution. A pH below 3.5 is not recommended, as it may affect the virus's stability.

A cost-effective tool for fast and automated screening of parameters during AAV purification process development is [CIM® SO3 Monolithic Well Plates]. With this product a lower sample amount is required, allowing several parameters (e.g., buffer compositions, pH values, salt types, and concentrations, etc.) to be studied in parallel. For example, to optimize mobile phase A (MPA), screen buffers with varying pH values (3.5 – 5.0) and concentrations of NaCl (0 mM – 750 mM), testing each condition with and without the addition of Poloxamer 188 (a detailed description of the experiment can be found in this application note). Purification results with CIM multi-well plates are directly comparable to the CIMmultus preparative line, as described in this article.

Scalability – Determining Optimal AAV Loading Density

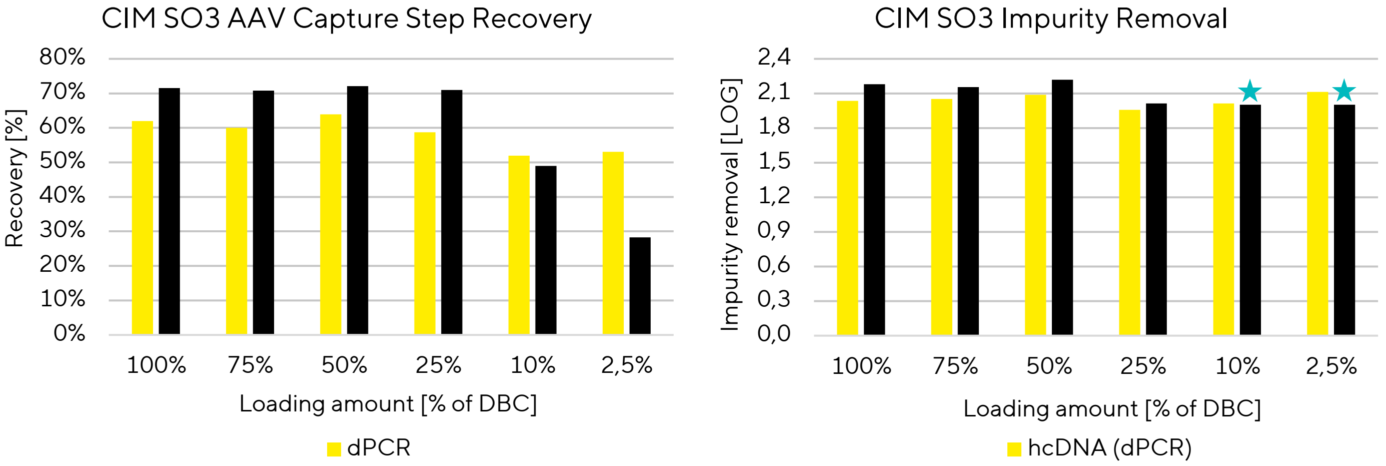
To determine the optimal AAV loading density range, it is important to verify the effect of different loading densities on recovery and impurity removal. A capacity run was performed using a clarified AAV8 harvest sample at seven loading density points, ranging from 9.1E+11 vp to 4.9E+13 vp/mL column (Figure 2). The capacity of clarified harvest material was determined at 3.6E+13 vp/mL column (teal line). Figure 2 shows that AAV elution using SO3 column occurs under consistent conditions, regardless of the AAV loading amount across a two-log AAV loading density range, demonstrating high scalability potential of CIMmultus SO3 columns.

Figure 2: *Chromatograms at various loading densities, using a CIMmultus SO3 1 mL column (vp/mL column): 4.9E+13 (134% DBC; orange line) 3.6E+13 (100% DBC; teal line), 2.7E+13 (75% DBC; turquoise line), 1.8E+13 (50% DBC; blue line), 9.1E+12 (25% DBC; dark purple line), 3.6E+12 (10% DBC; light purple line), 9.1E+11 (2.5% DBC; magenta line). Legend: absorbance at 280 nm (solid line), conductivity (dashed black line).*



Note: The DBC was determined using clarified harvest material. Pretreatment of clarified AAV lysate before entering the capture step will result in a higher loading capacity (see section Pre-capture step).

Graph 1: *Impact of loading amount on CIM SO3 AAV capture step recovery (left) and host cell impurity removal (right) (hcDNA and HCP) in the main elution fraction.*



The highest step recovery is achieved when loading 25% or more of the determined DBC. The loading amount does not significantly impact the removal of host cell DNA and HCP. With a loading of 10% and 2,5% of DBC (marked with teal star), the HCP values were below the limit of quantification (LOQ) due to high dilution, therefore the values are predicted. The LOQ of the Bradford method was determined to be 84 µg/mL. To prevent sample loss due to column overloading, we recommend loading less than 100% of DBC.

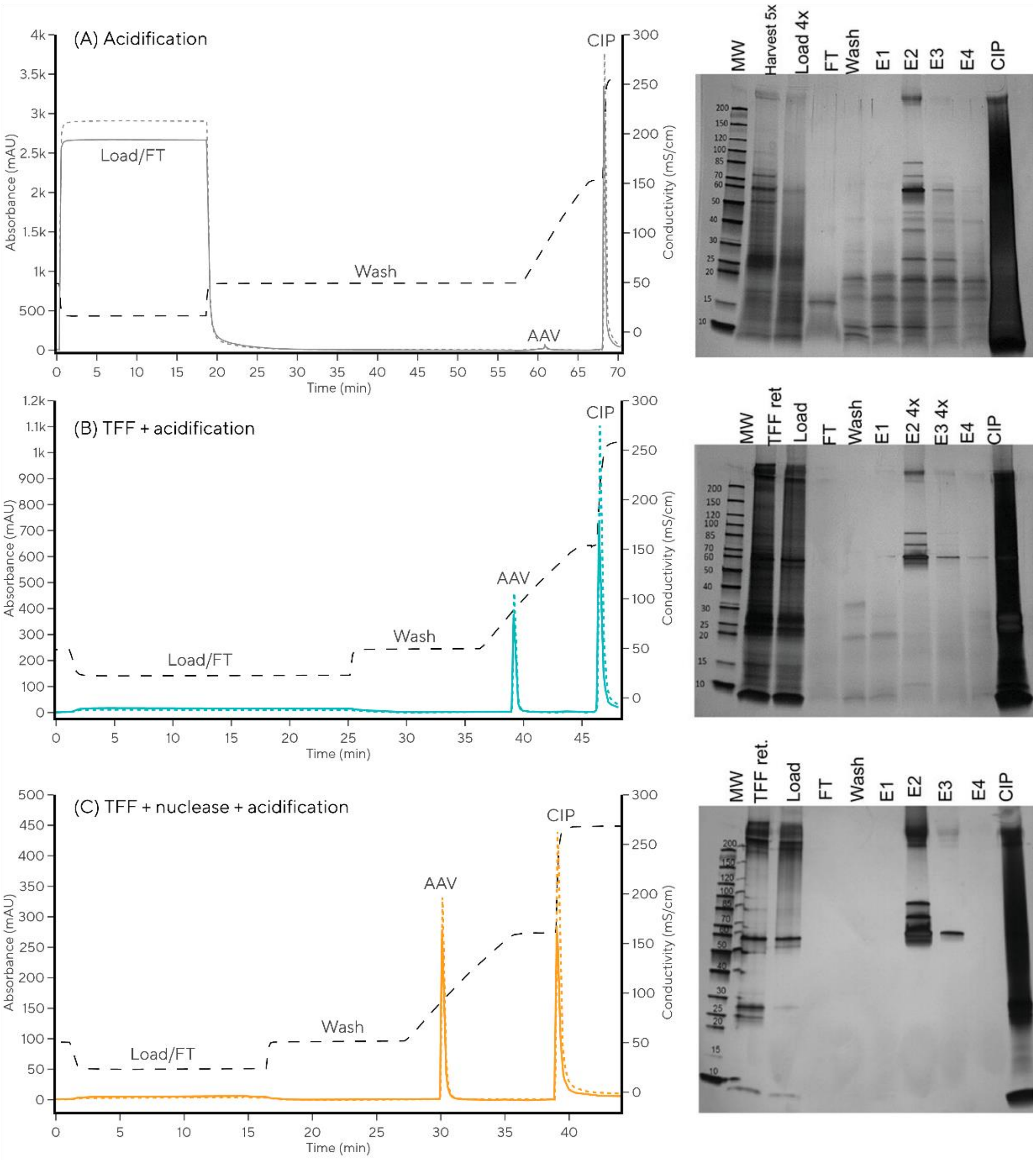
To summarize, monoliths are scalable solutions, ensuring consistent recovery and removal of critical impurities, such as host cell DNA and HCP. Column performance is expected to be maintained if loading at least 25% of DBC.

Pre-capture step: Pretreated Sample vs. Direct Application of Harvest to CIM SO3

To increase column's binding capacity and improve product purity, a pre-capture step can be implemented. For the pre-capture step TFF is usually used. However, for optimal results, it's advisable to explore a variety of pre-capture methods or even a combination of them, including OH chromatography, flocculation, TFF, solid phase extraction, precipitation and/or nuclease treatment.

To assess the impact of sample pretreatment on DBC, three different pre-capture approaches were evaluated in the following article: acidification (only acidified sample), TFF followed by acidification (TFF-acidified sample), and TFF coupled with salt-tolerant nuclease followed by acidification (TFF-nuclease treated-acidified sample) as shown in Figure 3.

Figure 3: *Effect of different sample pretreatments on the CIMmultus SO3 column capacity and AAV sample purity SDS-PAGE profile after CIM SO3 chromatography: (A) acidification (left) and an SDS–PAGE profile of chromatography fractions (right); (B) TFF with acidification (left) and an SDS–PAGE profile of chromatography fractions (right); (C) TFF coupled with nuclease treatment, followed by acidification (left) and an SDS–PAGE profile of chromatography fractions (right). Legend: absorbance at 280 nm (solid colored line); absorbance at 260 nm (dashed colored line); conductivity (dashed black line); CIP, cleaning in place; FT, flow-through; E1 – E4, elution fractions (E2 is the main elution fraction). Note that absorbance scale differs between the chromatograms.*



Clarified lysate AAV sample that was only acidified and filtered before application to CIMmultus SO3 column (Acidified only), a relatively small AAV elution peak was observed in comparison to the CIP peak. If the area peak ratio between the CIP peak and the AAV elution peak is substantially on the side of the CIP peak, it indicates that most of the binding sites on the CIMmultus SO3 column were occupied by impurities. This was confirmed by SDS-PAGE gel (Figure 3A). Pretreatment with TFF and acidification largely reduced amount of proteins before applying on the CIMmultus SO3, resulting in a higher AAV peak, compared with Acidified only sample. This resulted in better DBC and protein removal than acidification alone. However, some protein impurities remained, as shown in SDS–PAGE gel analysis (Figure 3B). Pretreatment with TFF coupled with salt-tolerant nuclease followed by acidification and filtration, effectively removed the majority of protein impurities, leading to a much higher AAV peak (Figure 3C). Nuclease treatment significantly improved the process efficiency. Results are summarized in the Table 1.

Table 1: *Determined DBC at 10% UV breakthrough, AAV recovery, and AAV purity results after three different pretreatments.*

		Acidification	TFF + acidification	TFF + nuclease + acidification
DBC (AAV vp/mL SO3)		1.2E+13	7.3E+13	1.4E+14
rAAV recovery (%)	Pretreatment	73.2	62.4	57.9
	Overall	56.9	49.9	50.9
Protein removal/E+13 vp (log removal)	Pretreatment	< LOQ	2.6	2.4
	Overall	2.6	3.8	3.8
DNA removal/E+13 vp (log removal)	Pretreatment	0.2	1.4	1.8
	Overall	1.5	2.3	2.1

Overall, the highest DBC and purity were achieved by implementing pre-capture step - TFF coupled with a nuclease, followed by acidification.

Scalability – From Linear to Step Elution

For a seamless transition from a linear gradient to a step elution, it's recommended to identify optimal elution conditions from the linear gradient elution. Once the pH value and salt concentration at which AAV elutes are determined, the appropriate salt molarity can be selected for the loading buffer to prevent impurities that are less positively charged than AAV from binding to the CIMmultus SO3 column.

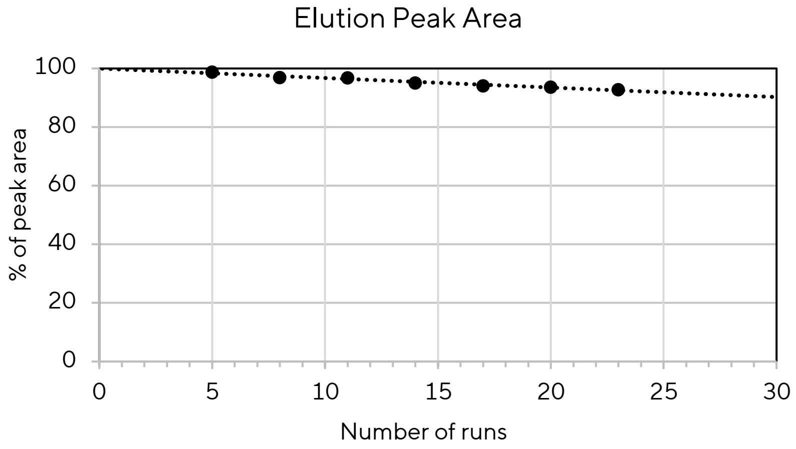
For large-scale manufacturing, step elution is frequently preferred due to its simplicity and efficiency. The step elution approach reduces buffer consumption and enhances control. This approach is not operator-dependent, ensuring that the volumes of fractions remain consistent across experiments. Implementation of step washes is described in this application note. To maintain a simplified and predictable process, it is important that the step washes are performed using individually prepared buffers based on the actual eluent concentration. Additionally, it is recommended to adjust the buffer temperature to the optimal working range during preparation and determine the exact mass of the titrant at a specific temperature.

Cleaning and Reusability

To improve cleaning, extend the contact time with cleaning and neutralization-equilibration solutions by reducing the flow rate (between 0.1 and 0.5 CV/min) or implement cleaning steps specific to the contaminants present in the sample.

According to our internal reusability study, after 10 runs, we observed a ~4% loss of peak area (i.e., less recovery) and a 10% loss after 30 runs (Graph 2). Between runs, column was cleaned with 1 M NaOH, 2 M NaCl for 0.5 h and neutralized with 5 CV of 1 M Ammonium acetate, pH 7.0 - 8.0. During this experiment, 5^{th} and after that each 3^{rd} run was performed with already SO3-purified AAV8 material. High robustness of column's performance after NaOH treatment contributes to lower costs of AAV manufacturing.

Graph 2*: Integrated elution peak area. Peak integration was done manually (integration interval for elution fraction was 3 CV).*



Conclusion

CIMmultus SO3 provides an advanced solution for capture step of any AAV serotype, ensuring high recovery rates and efficient impurity removal. Furthermore, it outperforms traditional affinity columns, offering superior results. CIMmultus SO3 demonstrated high reusability leading to lower purification cost. Additionally, faster processing time contributes to a lower overall cost of purification process, making CIMmultus SO3 the optimal choice.

Ordering Information

Cat No.	Product Name
311.6157-2	CIMmultus® SO3 1 mL Monolithic Column (Sulfonate) (2 µm channels)
414.6157-2	CIMmultus® SO3 4 mL Monolithic Column (Sulfonate) (2 µm channels)
411.6157-2	CIMmultus® SO3 8 mL Monolithic Column (Sulfonate) (2 µm channels)
614.6157-2	CIMmultus® SO3 40 mL Monolithic Column (Sulfonate) (2 µm channels)
611.6157-2	CIMmultus® SO3 80 mL Monolithic Column (Sulfonate) (2 µm channels)
814.6157-2	CIMmultus® SO3 400 mL Monolithic Column (Sulfonate) (2 µm channels)
811.6157-2	CIMmultus® SO3 800 mL Monolithic Column (Sulfonate) (2 µm channels)
1014.6157-2	CIMmultus® SO3 4000 mL Monolithic Column (Sulfonate) (2 µm channels)
1011.6157-2	CIMmultus® SO3 8000 mL Monolithic Column (Sulfonate) (2 µm channels)
904.6157-2	CIMmultus® SO3 4 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
901.6157-2	CIMmultus® SO3 8 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
914.6157-2	CIMmultus® SO3 40 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
911.6157-2	CIMmultus® SO3 80 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
924.6157-2	CIMmultus® SO3 400 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
921.6157-2	CIMmultus® SO3 800 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
934.6157-2	CIMmultus® SO3 4000 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
931.6157-2	CIMmultus® SO3 8000 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)
BIA-944.6157-2	CIMmultus® SO3 40000 mL cGMP Compliant Monolithic Column (Sulfonate) (2 µm channels)

FAQ

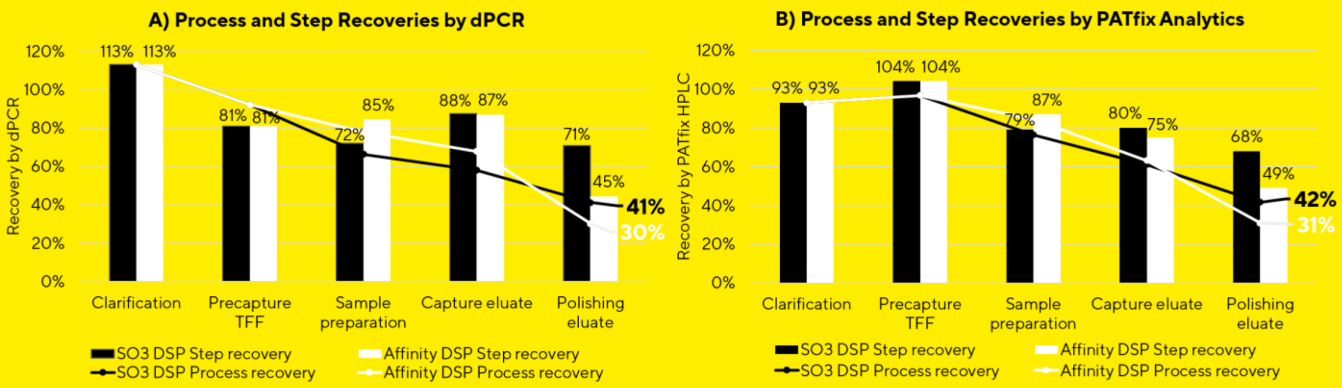
Where can I find product documentation?
Instructions for use and Product Certificates can be found at the following link: Product Documentation - Sartorius BIA Separations.

Does CIMmultus SO3 also separates empty and full AAV capsids?
No, CIMmultus SO3 does not separate empty and full AAV capsids. Additional polishing step is needed for full AAV enrichment. Widely used for this step are quaternary amine-based anion exchangers (AEX) like CIMmultus® QA HR. CIMmultus QA HR is a specialized monolithic column, which ensures consistent elution at the same chromatography conditions, regardless of the column size or batch. Alternative option are multimodal columns like CIMmultus® PrimaT.

Why is capture eluate buffer important, and how does it impact the polishing step?
Residual citric acid from the AAV capture step plays a significant role in non-reproducibility of the AEX polishing step. Before the polishing step, it is recommended to perform a buffer exchange or change the affinity eluate buffer (e.g. from citrate to a monovalent buffer, such as glycine or acetic acid). Further reading: The Story of Citric Acid and Non-Reproducibility in Empty/Full AAV Separation: What Could Go Wrong?

Why is it important to look at the overall DSP process recovery and not only at the step recoveries?
Although processes using CIMmultus SO3 and affinity capture show comparable step recoveries, the overall process recovery for the CIMmultus SO3 approach was 11% higher compared to the affinity approach, based on orthogonal dPCR and PATfix system analytics (Figure 4). The full process with the CIMmultus SO3 capture step resulted in a 30% increase in the number of doses available to the clinic compared to the full process with the affinity capture step (a detailed description of the experiment can be found in this poster).

Figure 4: *Comparison of step and process recoveries for both processes (A) Analyzed by dPCR. (B) Analyzed by Cation Exchange Chromatography Fingerprint (CEX-FP) on the PATfix System. The results shown in the step recoveries are the average values of the two repetitions.*



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