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Exploring Chromatographic Parameters for Enhanced Full AAV Enrichment With CIMmultus® PrimaT

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Abstract

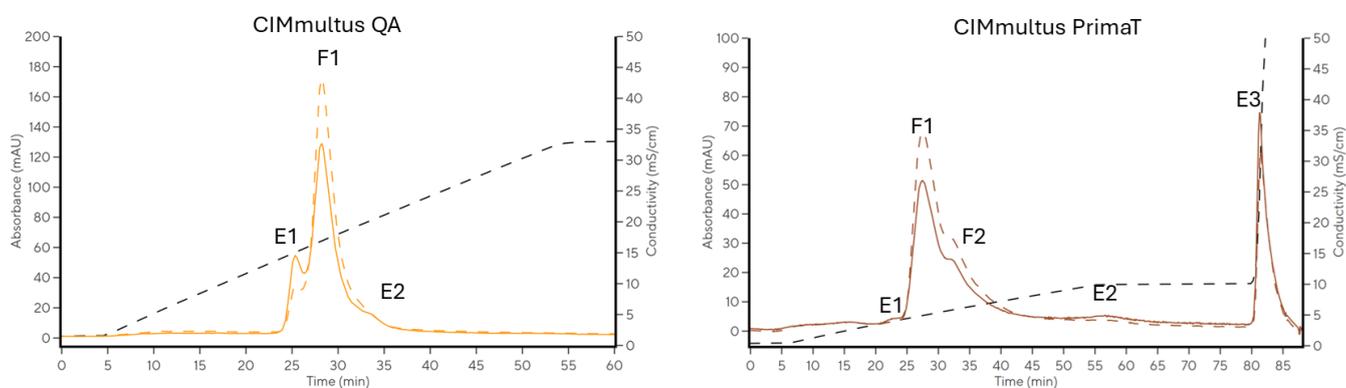
This application note explores the impact of various parameters on performance of CIMmultus® PrimaT column to optimize initial recommended methods, tailoring them to user's specific AAV sample for enhanced enrichment of full AAV. CIMmultus PrimaT is a multimodal column, allowing weak anion-exchange, hydrogen bonding and metal affinity coordination interactions. AAV serotype influences initial method selection. Screening different pH values critically impacts sample binding and alters elution profiles, affecting the separation of empty and full AAV capsids. The column's scalability is demonstrated through experiments on AAV loading density and transitions from linear to step elution gradients. CIMmultus PrimaT allows binding of all AAV serotypes and provides advanced removal of product-related impurities and AAV subspecies, while maintaining high recovery rates.

Introduction

The purpose of this application study is to showcase effect of different factors on the performance of the CIMmultus PrimaT column in downstream process development for enrichment of full AAV product.

CIMmultus PrimaT is a multimodal column, allowing weak anion-exchange, H-bond and metal affinity coordination interactions. Taking advantage of these properties, two individual methods were developed: Both Method 1 and Method 2 are described in detail in [Method guide: Enrichment of Full AAV With CIMmultus PrimaT](#). The main differences between the methods are different pH values during loading and elution steps. In contrast to monomodal anion exchangers (such as CIMmultus[®] QA HR), a decrease in pH value of buffers can promote binding of the AAV to the CIMmultus PrimaT column. CIMmultus PrimaT might provide a completely different elution profile compared to Quaternary amine-based chromatography columns, as shown in Figure 1.

Figure 1: Comparison of AAV subspecies separation by CIMmultus[®] QA (left) and CIMmultus PrimaT (right) column of 1 mL bed volume. Chromatography profile of AAV8 elution on CIMmultus PrimaT column using Method 2 (pH transition). CEX-SO3 purified sample with E/F ratio - 47% full AAV capsids loaded on the column at $2.7E+13$ vp/ mL column. Empty capsids (E) and full capsids (F) are labeled with numbers which indicate individual subspecies eluted from the corresponding column. Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).



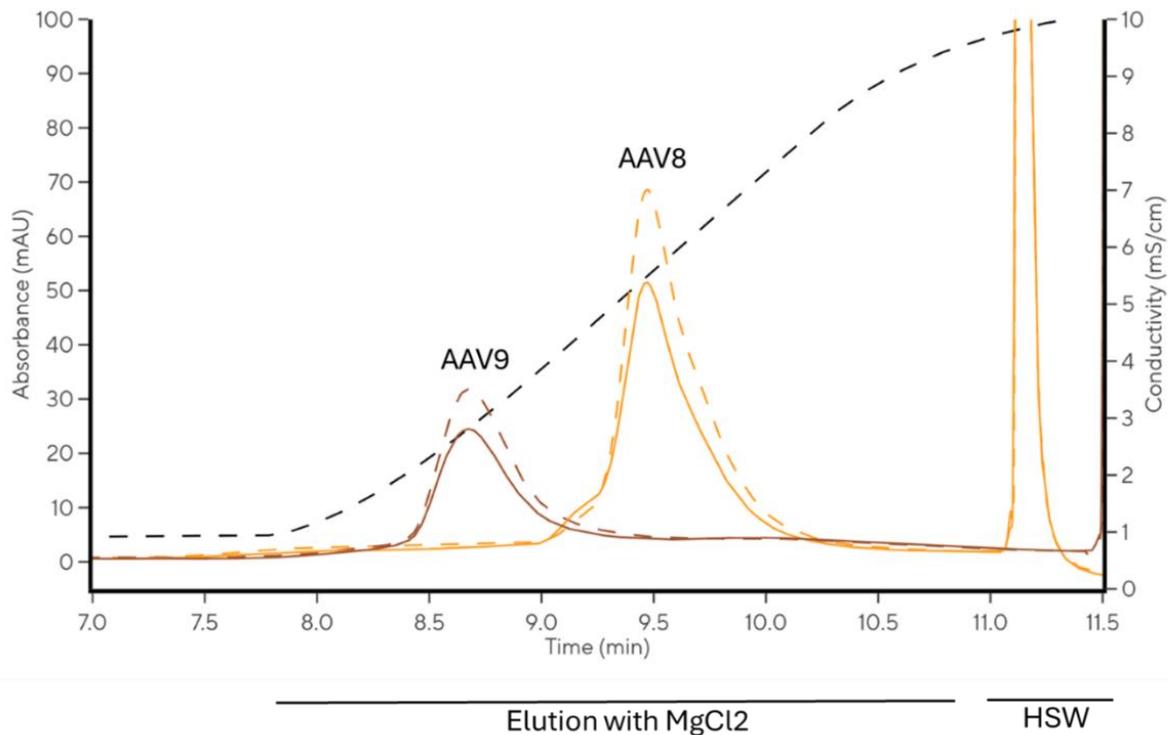
Similar to other CIMmultus monoliths, CIMmultus PrimaT is ready-to-use, scalable and caustic stable, enabling multi-cycling purification.

AAV samples exhibit considerable variability, which can affect purification results if always the same purification method is applied. To achieve optimal purification or to prove robustness of selected purification method, it is highly recommended to screen various chromatography conditions. When optimization is required, understanding the impact of different process parameters is crucial for effective method optimization.

AAV serotype

Information about the AAV serotype is an important attribute in selecting appropriate starting method, as binding interactions, elution profile pattern and conductivities at which AAV elute are largely influenced by the serotype. AAV8 typically elutes at higher conductivity values compared to AAV9. For AAV8 elution profile first peak is usually related to elution of empty AAV and second elution peak to elution of full AAV, while for AAV9 first elution peak is usually already related to full AAV elution (Figure 2).

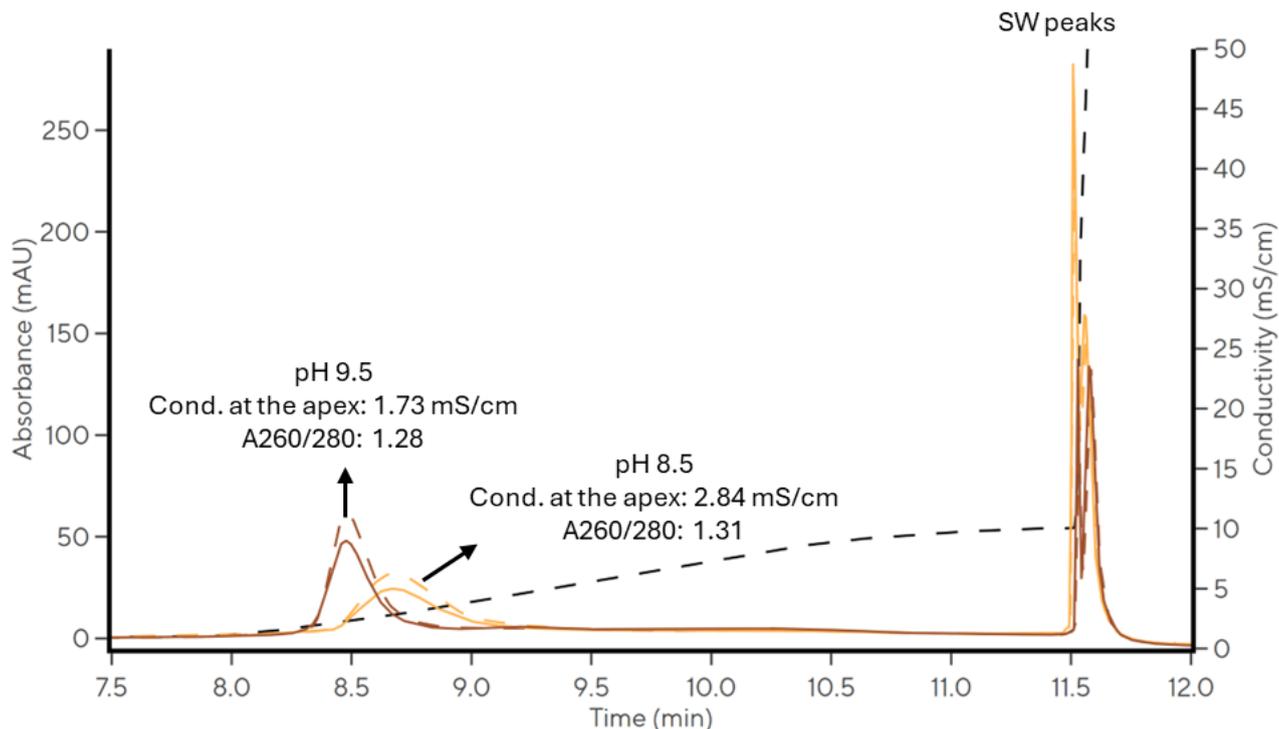
Figure 2: Chromatographic profile of the elution of AAV8 (orange) and AAV9 (brown) using CIMmultus PrimaT column following Method 1 (isocratic pH). CEX-SO3 purified sample AAV8 and AAV9 samples. E/F ratios: AAV8 = 47 %, AAV9 = 31 %. AAV loading amount: AAV8 = $2.7E+13$ vp/per mL column, AAV9 = $1.5E+13$ 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).



pH value

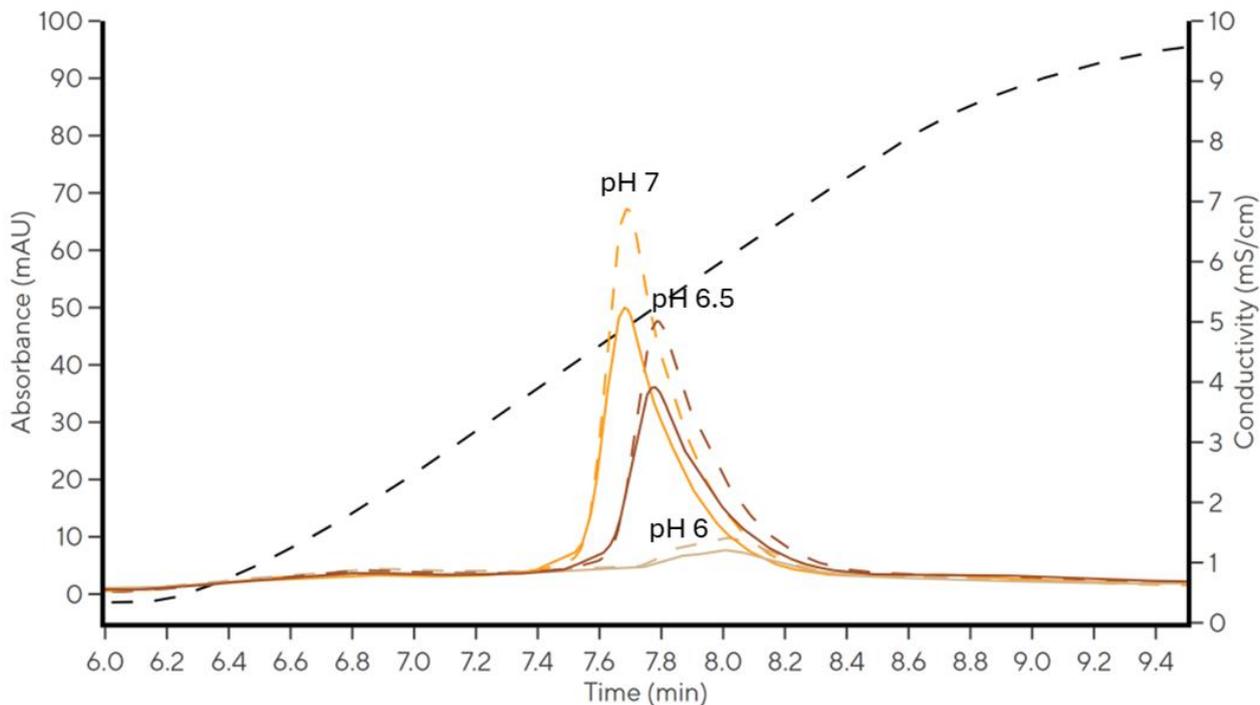
There are multiple interaction possibilities between AAV and the multimodal CIMmultus PrimaT column. Hydrogen bond and charge interactions are heavily influenced by pH values. Consequently, pH screening is a critical component of the purification process optimization. Figure 3 indicates that lowering the pH value of Method 1 to pH 8.5 promoted sample binding, with later elution, compared to pH 9.5. Additionally, at pH 8.5 more empty AAV capsids were present in salt wash, which increases full AAV capsid enrichment.

Figure 3: CIMmultus PrimaT purification following Method 1 on AAV9 sample at different pH values. Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).



If following Method 2, loading at pH of 7 is recommended. Reducing the pH below 7.0 (e.g. at pH 6.5) led to decreased binding and sample loss during application (Figure 4).

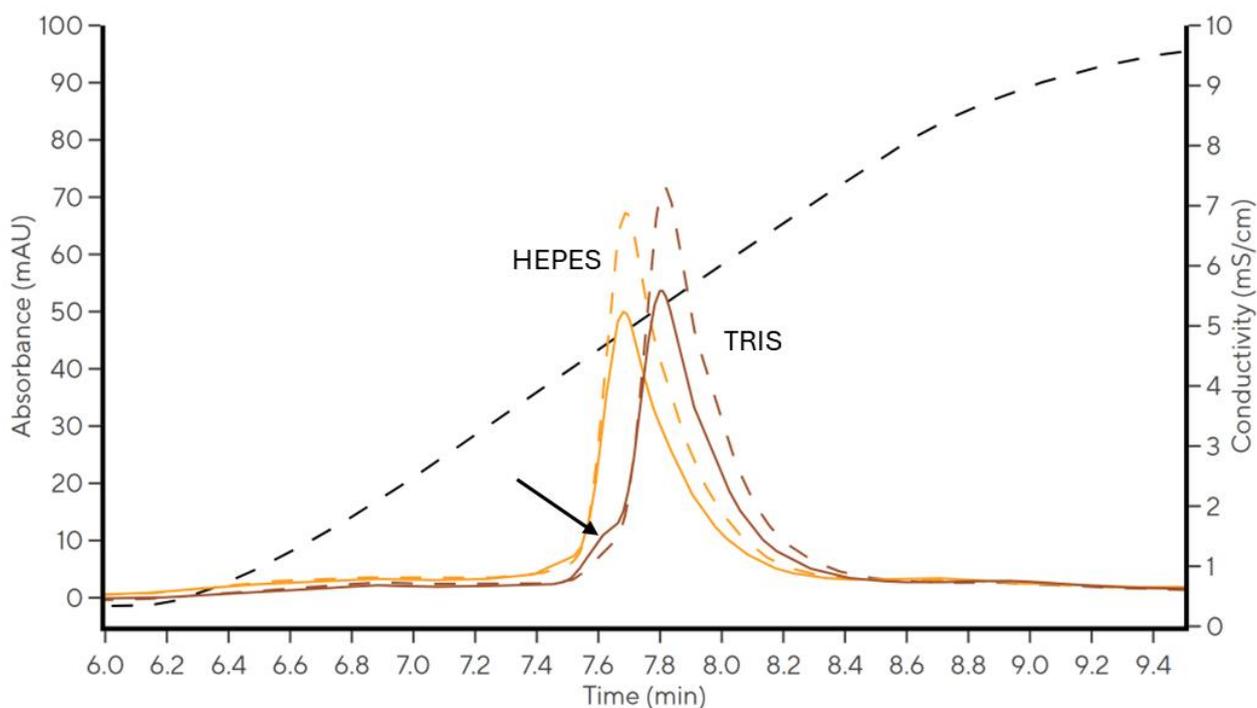
Figure 4: CIMmultus PrimaT purification following Method 2 on AAV8 sample at different pH values at loading step. Elution pH was constant for all three runs, maintained at pH 9.0. Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).



Buffering system

When optimizing purification by screening different pH values, selecting an alternative biological buffering system may be necessary to maintain buffer capacity at the chosen pH value. Figure 5 represents two commonly used buffers in bioprocesses – Tris and HEPES, resulting in slight differences of chromatographic profile.

Figure 5: CIMmultus PrimaT purification using Method 2 approach on AAV8 sample with different buffering systems used – HEPES (orange) and Tris (brown). A higher concentration of empty subspecies (black arrow) is obtained using Tris compared to HEPES for loading buffer. Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black). Arrow highlights the impurity peak.



Additives

Additives, that are known to affect multimodal CIMmultus PrimaT interactions, are expected to alter chromatographic elution profile of AAV empty-full separation. This can lead to migration of empty AAV from elution gradient to salt wash peak, different retention times, presence of AAV in flowthrough and diminished resolution between elution peaks. For highly reproducible CIMmultus PrimaT purification it is strongly recommended to buffer exchange loading sample in method specific equilibration buffer before loading. Figure 6 illustrates the negative effect if magnesium is present in the AAV sample during loading, while Figure 7 demonstrates effect of some other additives at high concentrations.

Figure 6: CIMmultus PrimaT purification of AAV8 sample following Method 2. Effect of magnesium chloride (Mg) in AAV sample during loading. Mg present in LOAD (brown), without Mg in LOAD (orange). Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).

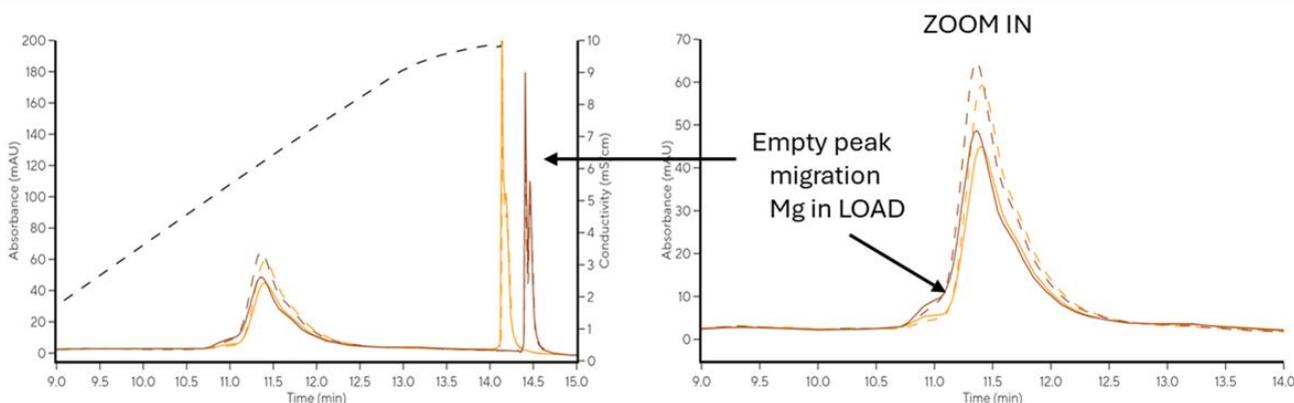
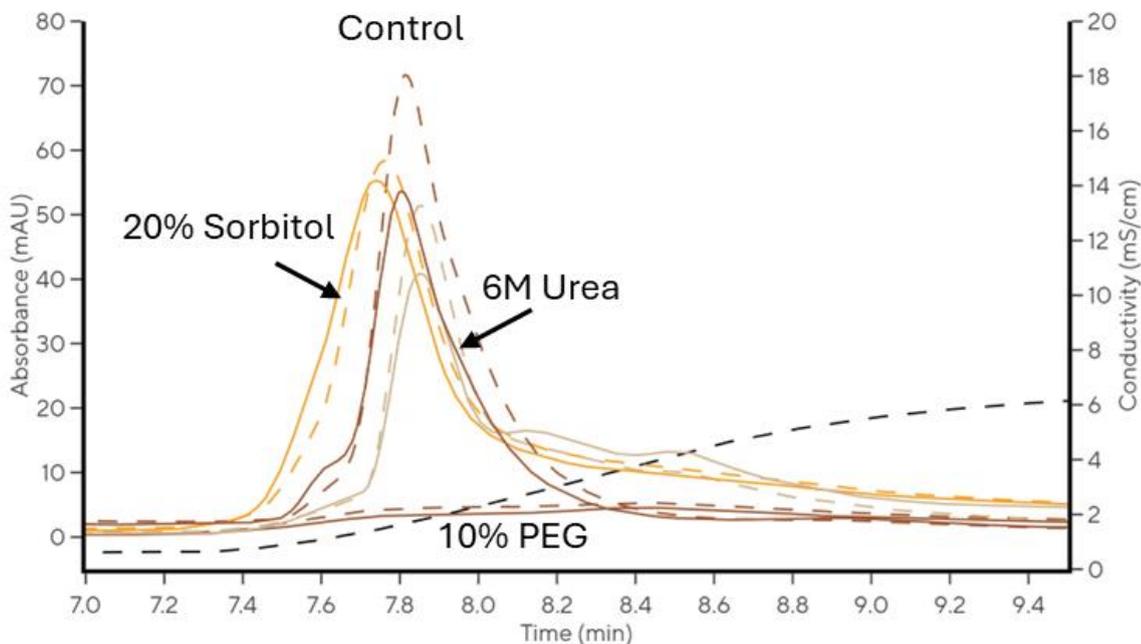


Figure 7: Effect of presence of Sorbitol, Urea and PEG in sample during loading to and elution from CIMmultus PrimaT. Purification of AAV8 sample using Method 2. Resolution and retention of elution peaks are reduced compared to control sample. Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).

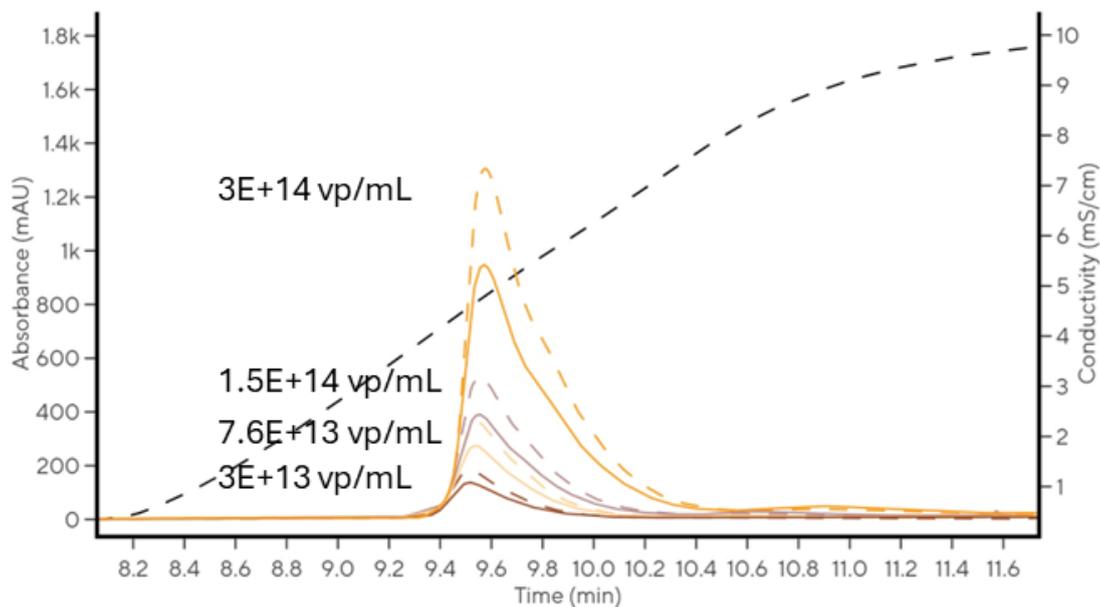


Scalability - AAV loading density

Effect of AAV loading density was tested with CIMmultus® SO3 purified sample with E|F ratio of 46% full AAV capsids at four loading points $1.4E+13$ vg - $1.4E+14$ vg/mL column (i.e. $3E+13$ vp - $3E+14$ vp/ml column). During this experiment column capacity was not reached. This experiment is aimed to verify the effect of different loading densities commonly used to determine optimal AAV loading range. Optimal loading range is defined by maintaining consistent retention times, conductivity values, resolution, and recovery regardless of the AAV loading density.

Figure 8 shows that the elution peak profiles remain consistent across different loading densities, with changing only the amplitude of elution peaks according to higher loading densities. The starting conductivity of peak elution for all four chromatograms has a 2% RSD and elution peak areas correlate linearly with $R^2=0.98$. Orthogonally to peak area ddPCR revealed an average step recovery of $73\% \pm 15\%$.

Figure 8: CIMmultus PrimaT chromatograms at various loading densities. $3E+14$ vp/mL column (ochre), $1.5E+14$ vp/mL column (violet), $7.6E+13$ vp/mL column (orange) and $3E+13$ vp/mL columns (brown). Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).



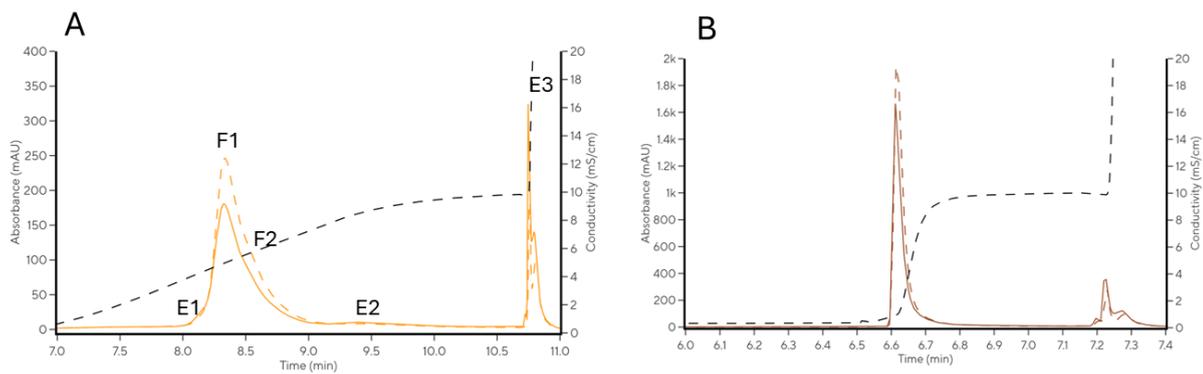
With CIMmultus PrimaT, consistent purification results were achieved across a tenfold variation in loading density. Since there are only up to fivefold increase in sizes of CIMmultus monolithic columns, such as scaling up from an 8 mL to 40 mL, scaling to the next size column with fivefold higher or lower loading densities within the optimal loading range should maintain reproducible separation performance, demonstrating high scalability potential of CIMmultus PrimaT columns.

Scalability - Implementation of step elution

Another key aspect of scalability is the seamless transition from a linear gradient to a step elution. Step elution approach reduces buffer consumption, simplifies the purification process and enhances control, making it the preferred method for large-scale GMP purification runs.

CIMmultus PrimaT special characteristics, such as its ability to strongly bind one subpopulation of empty AAV capsids that can only be stripped at high salt wash, enable straightforward implementation of step gradient elution for full AAV enrichment (Figure 9).

Figure 9: Linear gradient approach (A), step elution approach (B). AAV8 sample with 48% full AAV capsids ratio, loaded at $3.5E+13$ vg/ml of column, eluted by Method 2 approach. Step elution (B) was performed at 100% B1 buffer (50 m MgCl₂). Legend: Empty AAV subspecies (E1, E2 and E3), full AAV subspecies (F1 and F2). Legend: A260 (dashed line), A280 (solid line), conductivity (dashed black).



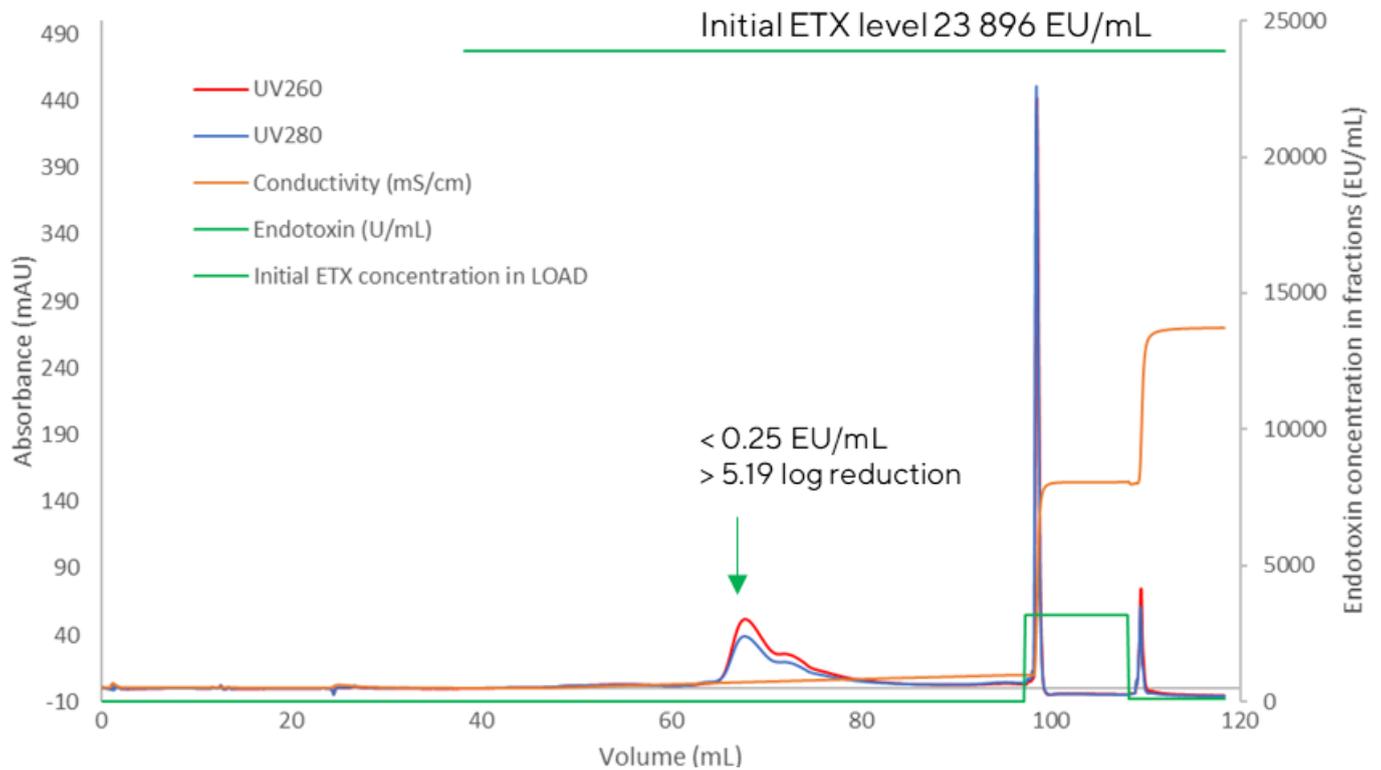
However, with only a single step elution fraction to a 100% B1 (Figure 9B), without any pre- or post-main eluate washes to selectively elute empty AAV subspecies (E1 and E2 in Figure 9A), step gradient delivered enrichment from 48% to 74 % full AAV capsids present in eluate.

Specific empty AAV subspecies (E1 and E2 fractions in Figure 9A) typically elute during MgCl₂ linear elution gradient. Therefore, to enhance the purity of full AAV target fraction by step elution approach, it might be necessary to implement more than one step elution wash within elution with ascending concentration of MgCl₂ to achieve a full AAV percentage comparable to that of the linear gradient elution with MgCl₂.

Analytical assessment - Endotoxin removal efficiency

AAV is usually produced in mammalian cell lines (e.g. HEK293) or in some cases in insect cell lines (e.g. SF9), generally resulting in low endotoxin levels. However, endotoxins (ETX) might be introduced at any step in the process. As CIMmultus PrimaT is used in the final purification step before sterile filtration, it is crucial to ensure that this step has high endotoxin removal capabilities. CIMmultus PrimaT column is highly efficient in reducing ETX due to its unique multimodal interaction characteristics, which ensure stronger binding of ETX to the column compared to binding of full AAV, eluting ETX during the salt wash strip.

Figure 10: Reduction of ETX with CIMmultus PrimaT Method 2 approach. Endotoxins were spiked to load fraction to a concentration of app. 24 k EU/mL. Main eluate shows final concentrations of below 0.25 EU/mL indicating reduction of more than 5 log.

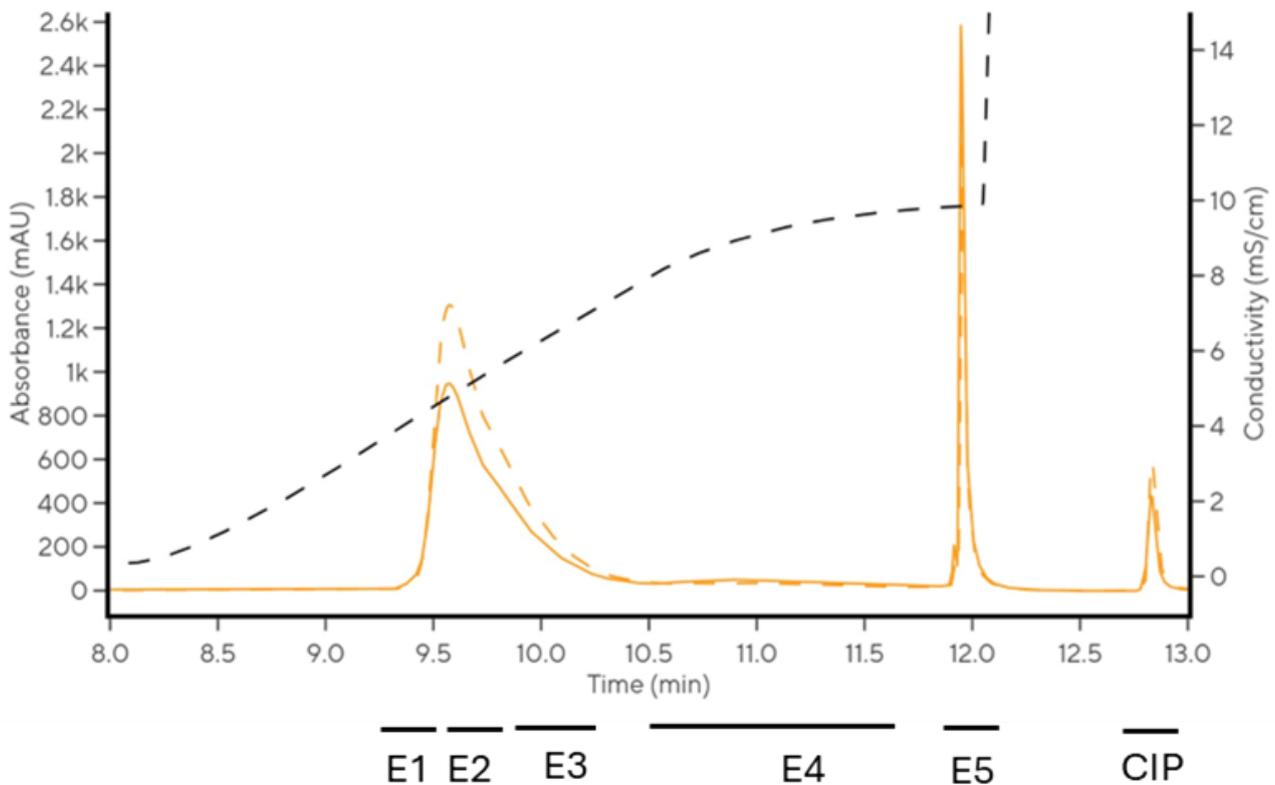


Analytical assessment - Subspecies separation

AAV capsids are known for their heterogeneity, even among capsids from the same serotype. Heterogeneity between samples from the same serotype can arise from various factors: Variability of AAV insert (e.g. different length of insert, scDNA or ssDNA), variability of AAV capsid (e.g. post-translational modifications due to differences in production batches, lysis or pre-polishing purification steps) etc. Therefore, relying only on charge differences using ion exchange columns might result in diminished or absence of resolution between the capsids subspecies. To overcome limitations posed by charge separation, CIMmultus PrimaT enables better AAV sub-species separation based on its ability for multimodal interactions with the AAV.

AAV 2/8 serotype sample, previously captured by cation exchange chromatography (CIMmultus SO3), was buffer exchanged in CIMmultus PrimaT loading buffer, following PrimaT purification Method 2 and loaded at $1.5E+14$ vg/mL of column. Purification resulted in separation of five individual elution peaks, marked as E1, E2, E3, E4 and E5 in the Figure 11. All these fractions were characterized by 4 different analytical techniques: HPLC AEX-FP-TRP, UC-HPLC, Cryo-TEM and Mass Photometry assays.

Figure 11: CIMmultus PrimaT purification run loaded at $1.5E+14$ vg/mL column. E1 to E5 represent fractions that were later assessed by orthogonal analytics. Legend: A260 (dashed line), A280 (solid line), conductivity (orange).



Characterization of individual CIMmultus PrimaT fractions by orthogonal analytics revealed that there were more than only empty and one full AAV species in elution fractions. At least three subpopulations of empty and two subpopulations of full AAV capsids were characterized. The difference between E2 and E3 predominantly full AAV fractions were confirmed by different analytical techniques (Figure 12 and Figure 13).

Figure 12: Ultracentrifugam overlay of Tryptophan (TRP) fluorescence (FLD) signals of individual fractions from CIMmultus PrimaT purification run. Fractions were formulated in solution of 3.0M CsCl and loaded at 3E+12 vg/vial (applies to genome containing fractions). Ultracentrifugation was performed on Sorvall WX90+ system at 245,000 g for 24h. After ultracentrifugation content of the vial was ran through PATfix® Tryptophan FLD detector.

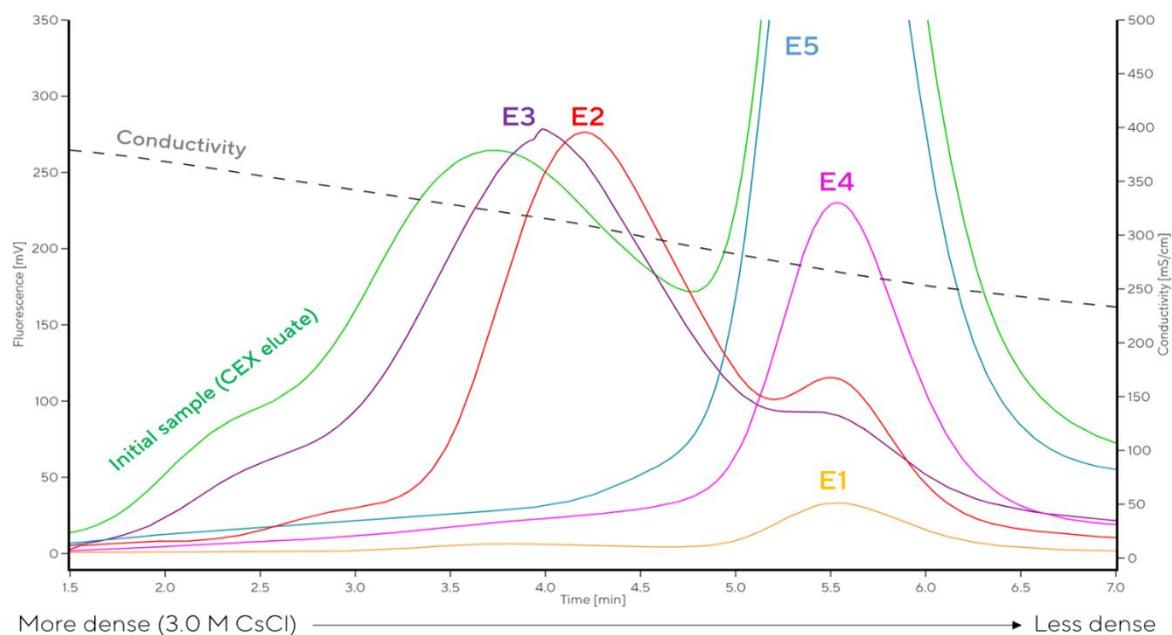
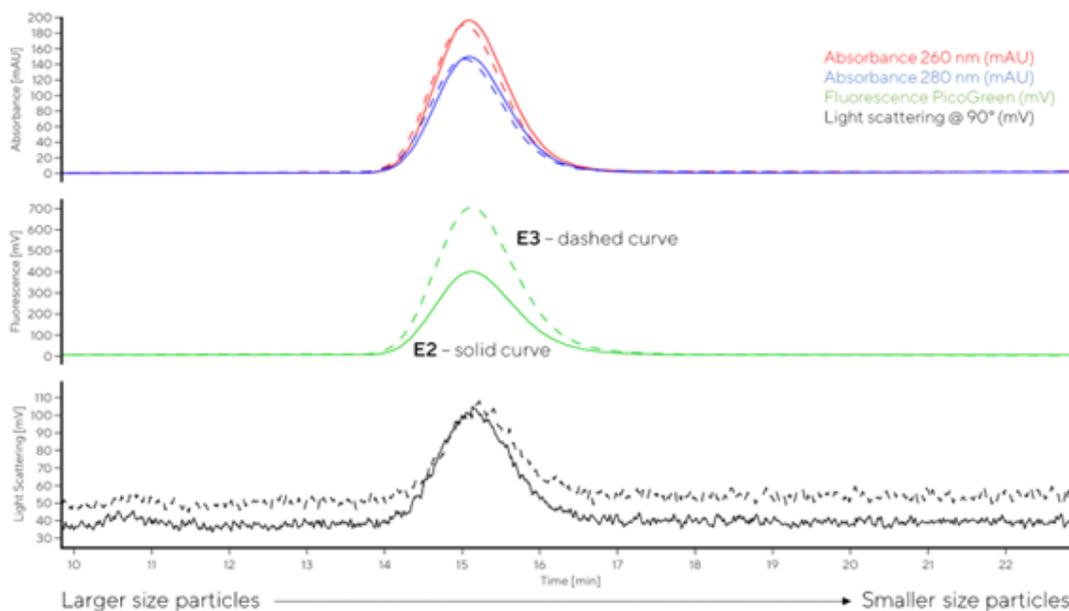


Figure 13: Chromatogram overlay of PATfix SEC analytics (SEC-FP-PG) with PATfix LC detectors (UV, FLD, MALS (Multi Angle Light Scattering)) E2 and E3 CIMmultus PrimaT fractions post ultracentrifuge (C). Fractions were stained with PicoGreen® incubated for 24h and applied to TSKgel G4000SWXL column, which enabled separation based on size differences and at the same time characterization of dsDNA presence.



Based on the ultracentrifuge result E3 fraction is denser and has higher electro-negative charge compared to E2 fraction. Moreover, although the same concentration of capsids, as perceived by MALS and UV signal, SEC-FP-PG assay points to a higher presence of dsDNA in case of E3 fraction. Entire study, including also Mass photometry, Cryo-TEM, HPLC-AEX analytics is available in the following [publication](#).

Conclusion

CIMmultus PrimaT demonstrated an advanced solution for enrichment of full AAV capsids, leveraging its unique multimodal interaction characteristics. These characteristics enable methods at neutral or near-neutral pH ensuring capsid stability, while allowing separation of multiple elution peaks and achieving high endotoxin clearance.

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