

## Improved Size-Exclusion Chromatography of Adeno-Associated Viruses (AAVs) With GTxResolve Premier BEH SEC 450 Å 2.5 µm Columns

---

Mateusz Imiolek, Szabolcs Fekete, Matthew A. Lauber

Waters Corporation

---

### Abstract

This application note discusses the use of GTxResolve™ Premier BEH™ SEC 450 Å 2.5 µm Columns for improved size-exclusion chromatography (SEC) analysis of adeno-associated viral (AAV) vectored gene therapies. SEC methods must be robustly developed to ensure that they provide an accurate picture of the aggregation profile of the drug product and they must be able to deliver reproducible results in a timely manner. Here, we introduce columns that have been purposefully designed and studied to ensure improved analyses of gene therapeutics (GTx) and AAV-based medicines. These columns are tailored for AAVs given their 450 Å average pore diameter particles and use of MaxPeak™ High Performance Surfaces (HPS). These technologies ensure effective fractionation of both high and low molecular weight size variants as well as improved recoveries of samples across a wider range of mobile phase conditions through the minimization of adsorptive losses. As such, serotype-independent AAV analysis is made possible with a simple 10 mM phosphate, 200 mM KCl mobile phase condition. Furthermore, we show that by using 2.5 µm particles, separation resolution can be significantly improved. Alternatively, it is shown that these 2.5 µm particles can be used to facilitate shorter analysis times without any loss of separation quality versus currently applied 5 µm columns. Finally, we tested the

chromatographic performance of GTxResolve Premier BEH SEC 450 Å 2.5 µm Columns in a guard-equipped configuration to demonstrate that high resolution separations can be achieved even when protecting the lifetime of the analytical column.

## Benefits

- Faster and higher throughput aggregate analyses
- More reliable analysis of high molecular weight species (HMWS) due to reduced secondary (non-specific) electrostatic interactions
- Good column stability and robustness of analyses with a single platform method and guard-equipped columns

---

## Introduction

Size exclusion chromatography (SEC) is a historical technique that has been widely applied in the biopharmaceutical industry for the measurement of aggregation and quaternary structures.<sup>1</sup> Not surprisingly, its use has also been adopted for the development and characterization of new gene therapy products, including AAV vectors for which aggregates are a critical quality attribute.<sup>2</sup> As a complex protein ensemble, AAV capsids can display a diverse aggregation profile, with a small part of self-associated forms reaching a sub-visible range of over 100 nm. The analysis of particles in this size regime requires the use of specialized analytical tools.<sup>3</sup> However, filled particles typically aggregate into smaller soluble dimeric and trimeric oligomers that can be routinely analyzed with SEC.<sup>3</sup> For this, an SEC column with particles having pores of around 450–500 Å serves well to efficiently separate monomer viral particles from both smaller impurities and higher order multimers.<sup>4</sup>

We previously demonstrated that a Waters GTxResolve Premier BEH SEC 450 Å Column packed with 3.5 µm particles can be used to achieve sensitive quantitation of AAV aggregates.<sup>3</sup> However, in this work, it was observed that mobile phase salt concentrations had to be individually optimized to ensure maximum recovery of dimer and trimer oligomers.

Proteins are known to interact with metal surfaces via electrostatic interactions. This leads to unwanted adsorption and peak distortion that has to be overcome by applying special considerations such as the use of a high ionic strength mobile phase. Just recently, this problem was successfully addressed for monoclonal

antibodies via the implementation of column hardware modified to have hydrophilic MaxPeak™ High Performance Surfaces (hHPS).<sup>3</sup> By using this type of low adsorption column hardware, method robustness and versatility was greatly improved. We predicted that a similar benefit could be obtained for AAV SEC by using hHPS hardware in BEH 450 Å SEC columns. The advantage of this column hardware is experimentally demonstrated in this application note.

Also demonstrated in this application note is the implementation of a 2.5 µm particle size. A decrease in particle size is predicted to increase the efficiency of analysis and enable more confident quantitation through better resolved aggregates. Moreover, the efficiency of a smaller particle size can be put to use to improve throughput and sensitivity.<sup>4</sup> Nevertheless, modern sub-3 µm particles have not yet been applied for the analysis of AAVs, presumably due to concerns about potential shearing and sieving effects.<sup>9</sup> In this application note, we show that GTxResolve Premier BEH SEC 450 Å Columns packed with 2.5 µm particles are, in contrast to original perceptions, well suited to performing AAV aggregate analyses. Most importantly, with their inherent efficiency advantages, these columns make it possible to perform higher throughput measurements and improve the resolution of size variants for the sake of more thorough characterization work.

---

## Experimental

### Sample and Mobile Phase Preparation

An aliquot of AAVs (AAV2, AAV5, or AAV9, 1E+13 vg/mL, Virovek, full containing CMV-GFP gene or empty) was transferred to a vial and directly injected into the SEC column.

*Note:* Caution should be taken if injecting highly aggregated samples. It is recommended to centrifuge down particulates and/or syringe filter such samples before injection onto an SEC column.

### LC Conditions

LC system:	ACQUITY™ H-Class Bio Plus (quaternary)
Detection:	Fluorescence detector: excitation: 280 nm, emission 350 nm; (2 points/s) and ACQUITY

	UPLC TUV detector with 5 mm titanium flow cell, 260 nm and 230 nm; (2 points/s)
Vials:	QuanRecovery™ with MaxPeak HPS 12 x 32 mm Screw Neck Vial, 300 µL, 100/pkg, (p/n: 186009186)
Column:	Analytical: GTxResolve Premier BEH SEC 450 Å 2.5 µm 4.6 x 150 mm Column (p/n: 186010584)
Guard:	MaxPeak GTxResolve Premier BEH SEC 450 Å 2.5 µm 4.6 x 30 mm Guard (p/n: 186010583)
Column temperature:	25 °C
Sample temperature:	6 °C
Injection volume:	1.0 µL
Flow rate:	0.25 mL/min
Mobile phase:	10 mM K <sub>2</sub> HPO <sub>4</sub> adjusted with HCl to pH 7.4, 200 mM KCl, 0.2 µm sterile filtered  (Mobile phase used for only 2 days or otherwise made with azide to impede bacterial growth).

---

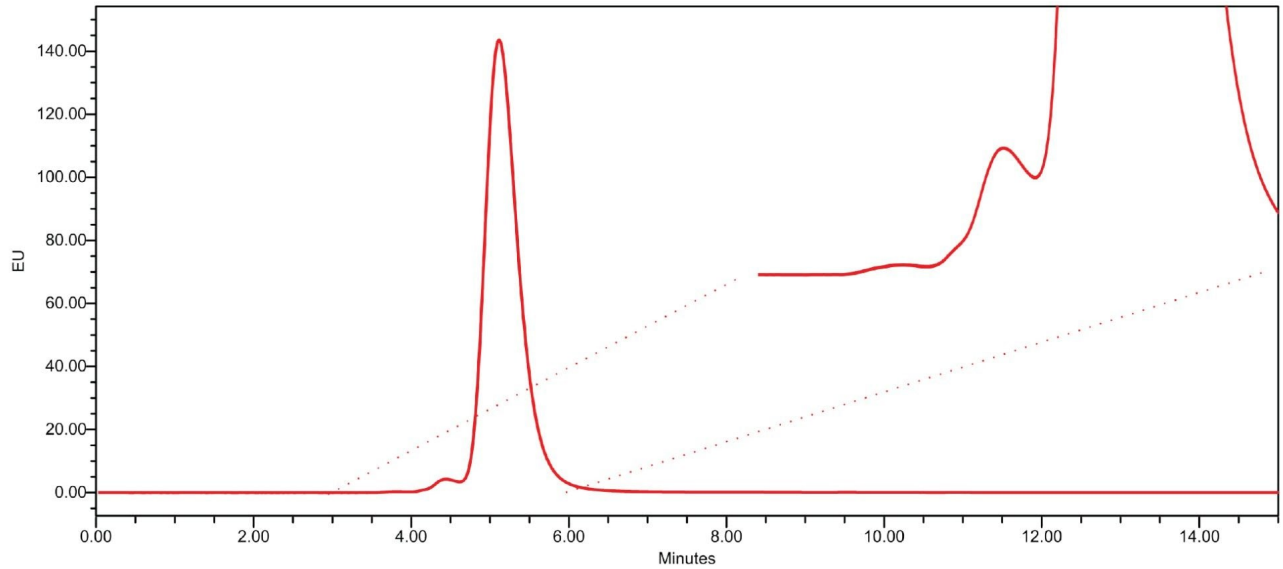
## Results and Discussion

### Particle Size and Kinetic Performance

To understand the performance of a typical AAV analysis, we chose to first investigate separations with silica 5 µm packing material. PEEK hardware was employed and an example separation of an AAV2 sample was

obtained as shown in Figure 1A. The use of a 0.25 mL/min flow rate resulted in a reasonable  $t_0$  time of < 10 minutes. However, we were only able to achieve partial resolution of the HMWS peaks. Under these conditions, the main peak eluted with  $N = 630$  USP plates and the analysis had unsatisfactory efficiency. A lengthier run would be needed to achieve the required resolution (i.e. a run with a longer column or a lower flow rate). Next, we reran the separation using an GTxResolve Premier BEH SEC 450 Å 2.5  $\mu\text{m}$  Column of the same dimensions (Figure 1B). This analysis yielded a much improved separation of the oligomeric species with a similar  $t_0$  time of < 10 minutes and significantly higher plate numbers for the main peak ( $N = 2760$ ). That is, the GTxResolve Premier BEH SEC 450 Å 2.5  $\mu\text{m}$  Column exhibited a four times more efficient AAV separation than the 5  $\mu\text{m}$  particle column. The particle size difference would be expected to confer a two fold efficiency gain. It is possible that the extra gain in efficiency can be attributed to differences in the quality of column packing.

**A** PEEK Hardware  
5  $\mu$ m Silica 500Å Particle Column



**B** XBridge Premier GTx  
BEH SEC 450Å 2.5  $\mu$ m Particle Column

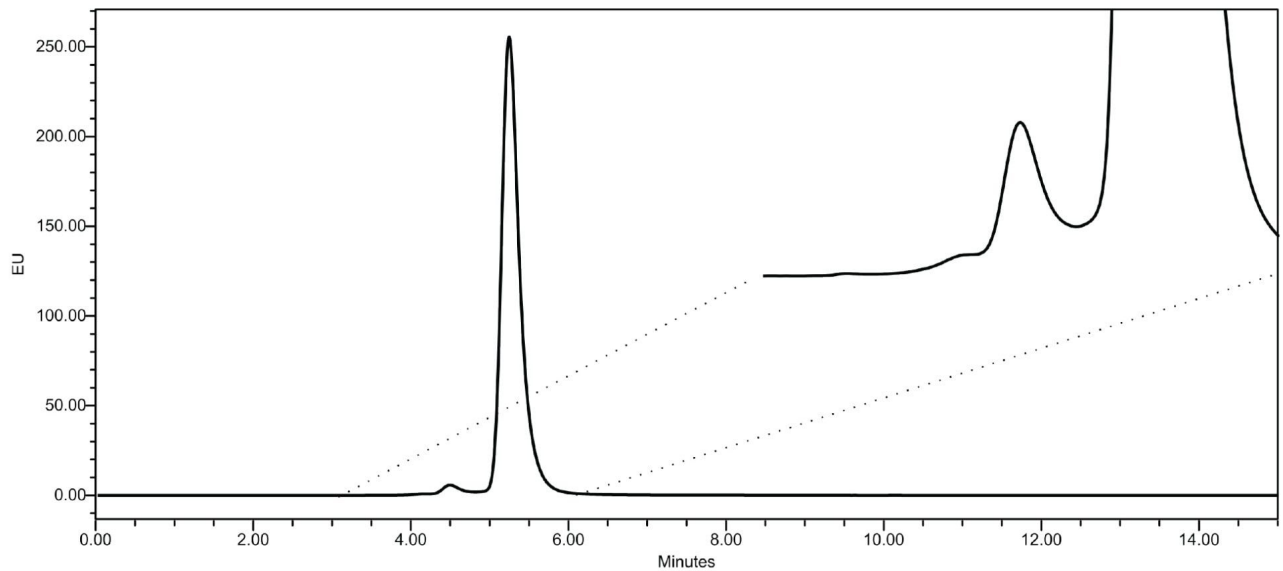


Figure 1. SEC separation of an AAV2 sample using A) a PEEK hardware 4.6 x 150 mm column packed with 5  $\mu$ m 500 Å silica particles and B) an GTxResolve Premier BEH SEC 450 Å 2.5  $\mu$ m, 4.6 x 150 mm Column. The HMWS region of the chromatogram is enlarged to show differences in the compared separations.

## The Impact of Column Hardware

The majority of commercially available columns are constructed from stainless-steel hardware, which is known to be prone to unwanted secondary interactions with analytes. In order to assess this impact on viral vector analysis, we compared two types of column hardware using the AAV2 sample, namely a silica 5  $\mu\text{m}$  stainless-steel column versus a hydrophilic HPS 2.5  $\mu\text{m}$  column (the GTxResolve Premier BEH SEC). A mobile phase comprised of a standard 200 mM salt concentration and a pH of 7.4 was employed. Under these conditions, almost no aggregates could be detected using the stainless-steel column, suggesting loss of HMWS to the metal surfaces (Figure. 2). In contrast, the GTxResolve Premier BEH SEC showed high apparent recoveries of the HMWS peaks. Laborious method development was required to observe comparable aggregates levels on both columns.

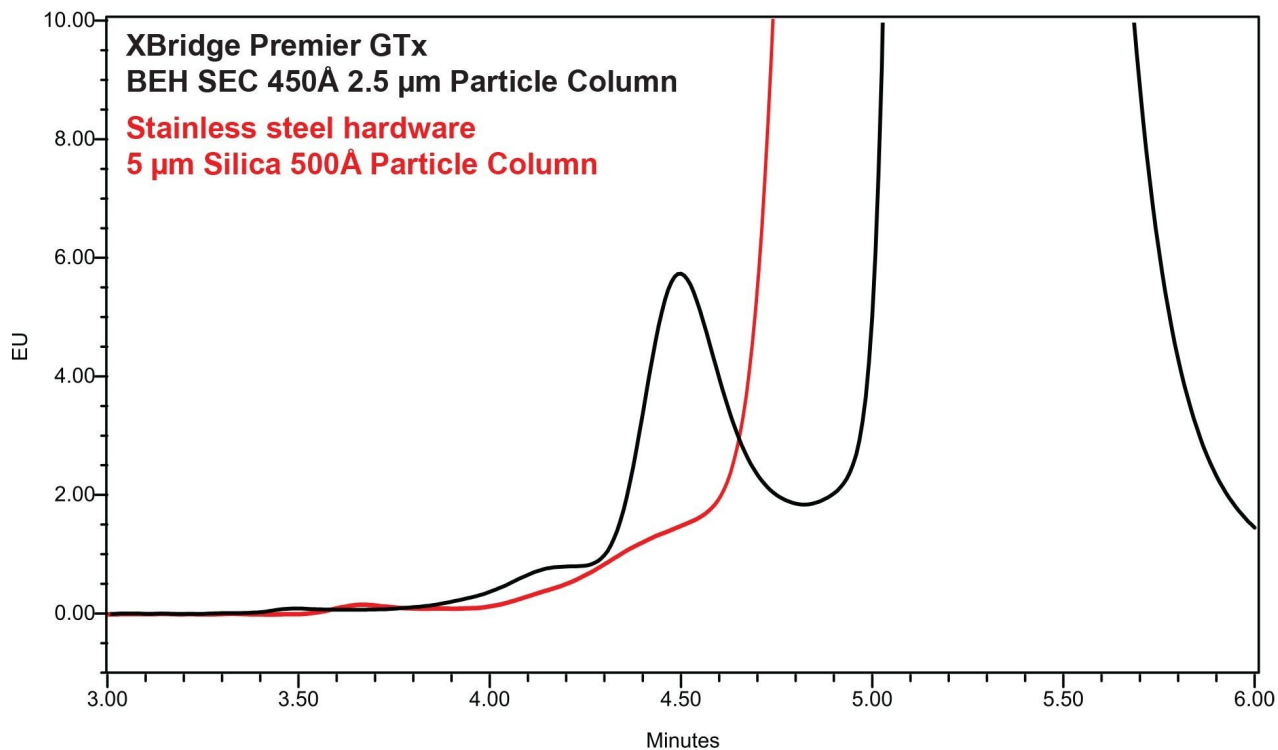


Figure 2. Zoomed views of an AAV2 chromatogram as obtained with stainless- steel hardware (4.6 x 150 mm, 5 µm particle, red trace) versus hHPS hardware (GTxResolve Premier BEH SEC 450 Å 2.5 µm 4.6 x 150 mm Column, black trace). Separations were performed with a mobile phase containing a standard ionic strength buffer (10 mM phosphate pH 7.4 + 200 mM KCl).

## Speeding up the Analysis and Performing Fast Separations

We next aimed to show that the GTxResolve Premier BEH SEC 450 Å 2.5 µm Column can generate SEC chromatograms of various AAV serotypes in significantly reduced time. We chose to run the column at its maximum recommended flow rate of 0.6 mL/min, which led to a 5-minute analysis time (Figure 3). With these conditions, we were able to quantify aggregates at levels that proved to be in agreement with data from low flow rate runs on the reference 5 µm PEEK hardware column (<15% difference, Table 1). To obtain comparable resolution on the reference column, separations needed to be run at 0.05 mL/min resulting in a ten times longer analysis of 50-minutes.



Flow rate (mL/min)	Column	HMWS quantification (%)			
		AAV2	AAV9	AAV5 - empty	AAV5 - full
0.60	XBridge Premier GTx	2.2	9.5	2.7	3.6
0.25	BEH SEC 450Å 2.5 µm Column	2.4	9.0	2.2	4.4
0.25	PEEK Hardware Silica 500Å	2.1	8.3	2.3	3.3
0.05	5 µm Column	2.5	10.6	2.8	4.2

Table 1. Quantification of HMWS (%) with GTx BEH and standard column at different flow rates.

**XBridge Premier GTx  
BEH SEC 450Å 2.5 µm Particle Column  
Flow rate = 0.6 mL/min  
Analysis time = 5 min**

**PEEK hardware  
5 µm Silica 500Å Particle Column  
Flow rate = 0.05 mL/min  
Analysis time = 50 min**

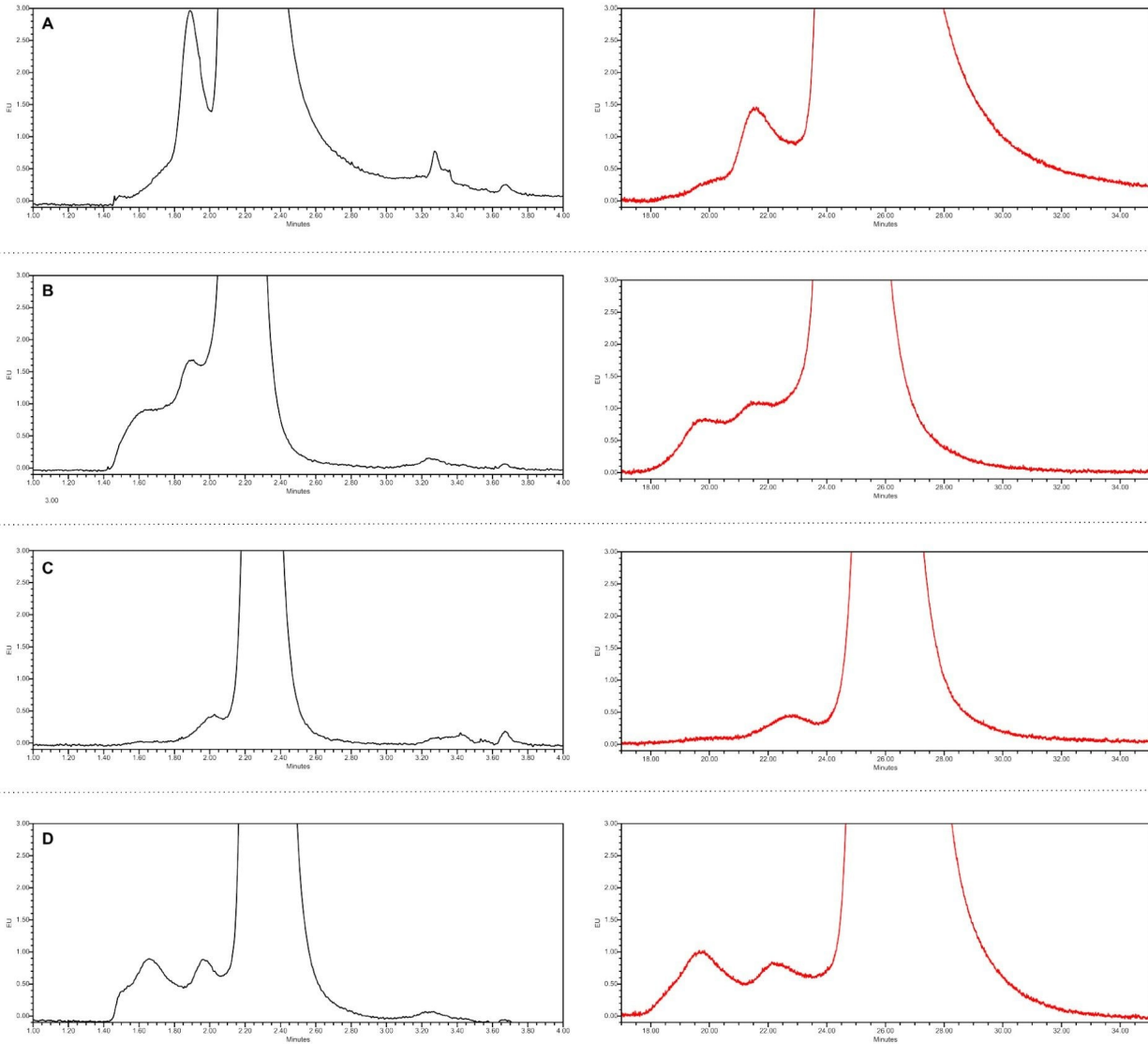
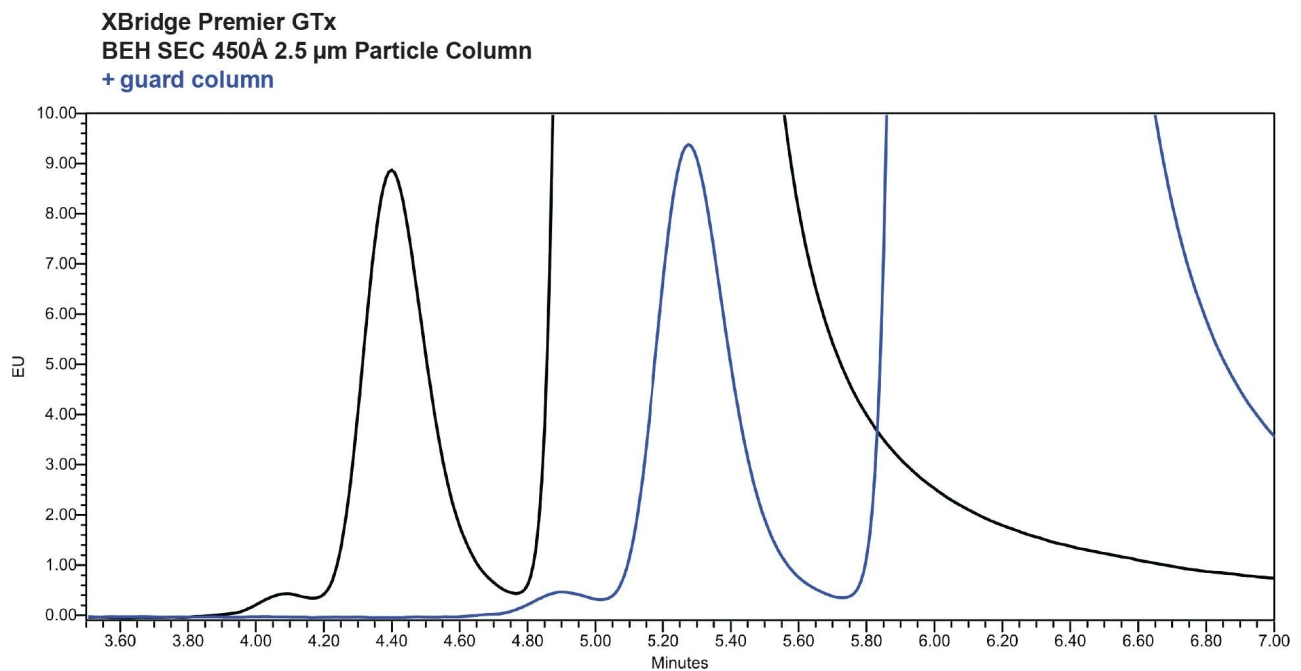


Figure 3. Zoomed views of SEC chromatograms for A) AAV2, B) AAV9, C) AAV5 – empty, D) AAV5 – full samples as obtained with an GTxResolve Premier BEH SEC 450 Å 2.5µm Column operated at 0.6 mL/min (2.5 µm particle, 5 minute analysis time, black traces) and a reference 500 Å column at 0.05 mL/min (5 µm particle, 50 minute analysis time, red traces).

## On the Use of Guard Columns

Reliable and reproducible analysis of AAVs requires unchanged performance of the analytical column over long periods of use. Considering the complex nature of viral particles, it is envisioned that the injections of highly aggregated samples might overtime cause fouling at the column inlet. With that, overall performance of the analysis will gradually decline. Therefore, it is recommended to make use of short, guard columns in order to extend the lifetime of the analytical column. We compared the efficiency of separation for several AAVs on the GTxResolve Premier BEH SEC 450 Å 2.5 µm Column used on its own versus being equipped with a guard (Fig. 4). While the time of analysis is minimally extended (20%), the resolution of analysis is also improved (Rs 2.1 vs 1.9), as expected for an SEC separation with an effectively longer column (30 mm guard plus 150 mm analytical column). It should be noted that this guard is designed for use with both 4.6 and 7.8 mm ID analytical columns.



*Figure 4. Zoomed view SEC chromatograms for AAV2 as obtained using an GTxResolve Premier BEH SEC 450 Å 2.5 µm Column equipped (blue trace) and not equipped (black trace) with a 4.6 x 30 mm guard. HMWS content was measured to be 1.7% in both cases. The guard was removed after > 40 AAV injections, and the analysis was repeated with just the analytical column to obtain the black trace chromatogram.*

---

## Conclusion

This application note demonstrates the utility of an GTxResolve Premier BEH SEC 450 Å 2.5 µm Column for the analysis of AAV particles and quantitation of its self-associated oligomeric aggregates. Superior kinetic efficiency of this column was evident when compared to a reference 5 µm particle column. The implementation of MaxPeak Premier HPS technology in the GTxResolve Premier BEH SEC significantly reduced secondary interactions and enabled efficient separations of various AAV serotypes using the same conditions. Such an experiment condition was ineffective with a standard stainless-steel column due to poor recovery of HMWS peaks. Importantly, decreased particle size and high flow rates were found to have no impact on the measurement of aggregation profiles. In addition, ten-fold faster analysis times could be achieved without a loss in resolution by increasing the flow rate of the method. Lastly, a guard column was shown to be compatible with AAV analysis.

---

## References

1. P. Hong, S. Koza, E.S.P. Bouvier, A Review, Size Exclusion Chromatography for the Analysis of Protein Biotherapeutics and Their Aggregates, *J. Liq. Chromatogr. Relat. Technol.* 35 (2012) 2923–2950. <https://doi.org/10.1080/10826076.2012.743724> <<https://doi.org/10.1080/10826076.2012.743724>> .
2. Gimpel AL, Katsikis G, Sha S, *et al.* Analytical Methods for Process and Product Characterization of Recombinant Adeno-Associated Virus-Based Gene Therapies. *Mol Ther Methods Clin Dev.* (2021);20:740–754. <https://doi:10.1016/j.omtm.2021.02.010> <<https://pubmed.ncbi.nlm.nih.gov/33738328/>> .
3. Deng C. AN2003: Quantifying AAV Aggregation and Quality Attributes by FFF-MALS, Wyatt Technology Application Note, [https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2003-quantifying-AAV-aggregation-and-CQAs-by-FFF-MALS\\_CDeng.pdf](https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2003-quantifying-AAV-aggregation-and-CQAs-by-FFF-MALS_CDeng.pdf) <[https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2003-quantifying-AAV-aggregation-and-CQAs-by-FFF-MALS\\_CDeng.pdf](https://wyattfiles.s3-us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2003-quantifying-AAV-aggregation-and-CQAs-by-FFF-MALS_CDeng.pdf)>
4. Bartalis J, Chen M., AN2004: Why and How to Quantify Aav Aggregates by Fff-Mals, [https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2004-How\\_to\\_quantify\\_AAV+aggregates\\_by\\_FFF-MALS.pdf](https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2004-How_to_quantify_AAV+aggregates_by_FFF-MALS.pdf) <[https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2004-How\\_to\\_quantify\\_AAV+aggregates\\_by\\_FFF-MALS.pdf](https://wyattfiles.s3.us-west-2.amazonaws.com/literature/app-notes/fff-mals/AN2004-How_to_quantify_AAV+aggregates_by_FFF-MALS.pdf)>

5. McIntosh, N.L., Berguig, G.Y., Karim, O.A. *et al.* Comprehensive Characterization and Quantification of Adeno Associated Vectors by Size Exclusion Chromatography and Multi Angle Light Scattering. *Sci Rep* 11, 3012 (2021). <https://doi.org/10.1038/s41598-021-82599-1>. <<https://www.nature.com/articles/s41598-021-82599-1>>
6. Koza MK, Chen W. Size-Exclusion Chromatography Analysis of Adeno-Associated Virus (AAV) Preparations Using a 450 Å Diol-Bonded BEH Column and Fluorescence Detection. Waters Application Note: [720006812](#), March 2020.
7. Kizekai L, Shiner S, Lauber MA. Waters ACQUITY and XBridge Premier Protein SEC 250 Å Columns: A New Benchmark in Inert SEC Column Design. Waters Application Note: [720007463](#), 2022.
8. Goyon A, Beck A, Colas O, Sandra K, Guillaume D, Fekete S. Evaluation of Size Exclusion Chromatography Columns Packed With Sub-3µm Particles for the Analysis of Biopharmaceutical Proteins. *J Chromatogr A*. 2017 May 19;1498:80–89. doi: [10.1016/j.chroma.2016.11.056](https://doi.org/10.1016/j.chroma.2016.11.056) <<https://pubmed.ncbi.nlm.nih.gov/27262372/>> .
9. [Meng H, Sorrentino M, Woodcock D, O'Riordan C, Dhawan V, Verhagen M, Davies C. Size Exclusion Chromatography with Dual Wavelength Detection as a Sensitive and Accurate Method for Determining the Empty and Full Capsids of Recombinant Adeno-Associated Viral Vectors. *Hum Gene Ther*. 2022 Feb;33(3-4):202–212. <https://www.liebertpub.com/doi/10.1089/hum.2021.123> <<https://www.liebertpub.com/doi/10.1089/hum.2021.123>> . <<https://www.liebertpub.com/doi/10.1089/hum.2021.123>>

---

## Featured Products

[ACQUITY UPLC H-Class PLUS Bio System <https://www.waters.com/10166246>](https://www.waters.com/10166246)

[ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>](https://www.waters.com/514228)

[Empower Chromatography Data System <https://www.waters.com/10190669>](https://www.waters.com/10190669)

720007961, June 2023



© 2024 Waters Corporation. All Rights Reserved.

[Terms of Use](#) [Privacy](#) [Trademarks](#) [Careers](#) [Cookies](#) [Cookie Preferences](#)