

Biotechnology

Size exclusion chromatography with fluorescence detection to determine AAV titers following downstream processing

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Keywords

Adeno associated virus (AAV), cell and gene therapy, product titer, downstream processing, liquid chromatography (LC), size exclusion chromatography (SEC), fluorescence detection

Key benefits

- Size exclusion chromatography (SEC) following downstream processing is shown to be a rapid and cost-efficient method for the determination of product titers.
- Employing fluorescence detection provides high sensitivity, thus reducing the required sample amounts.

Goal

To demonstrate the suitability of SEC for a fast and reproducible evaluation of adeno-associated virus (AAV) product titers

Introduction

Gene therapy using viral vectors, such as AAV, is a rapidly evolving area of biopharmaceutical research with multiple clinical studies showing promising results in treating severe disease.¹ However, despite this great advancement, manufacturing as well as downstream processing and analytical characterization of AAVs remains challenging mainly due to low sample yields, long turnaround times for established methodologies, and serotype complexity.^{2,3}

AAVs consist of a protein capsid with an average molecular weight of 3.5–4.0 MDa, containing a therapeutic single-stranded DNA genome. There are several cellular expression systems for recombinant AAV production with the most common ones being mammalian HEK293 cells or SF9 insect cells.⁴ The inherent complexity of recombinant protein manufacturing requires detailed monitoring of various product quality attributes (PQAs), such as correct capsid assembly and packaging but also the assessment of obtained product titers. Knowing the exact titer is not only important to monitor production efficiency in general but also to determine the correct dosage for subsequent treatment.

Here, we demonstrate how size exclusion chromatography can be used to provide a cost-efficient and precise method to rapidly determine AAV product titers following a single-step affinity purification.

In the past, several absorbance-based methods for viral vector titer determination have been proposed,^{5,6} but they have been heavily affected by remaining contaminants such as host cell proteins or DNA. SEC using Thermo Scientific™ MAbPac™ SEC-1 columns efficiently separates AAV capsids from lower molecular weight compounds. It therefore enables precise analysis of the viral capsid concentration. To overcome the common issue of limited sample availability, we employ highly sensitive fluorescence detection, which allows analysis of samples at low concentrations. Therefore, the proposed workflow successfully enables product characterization at various production steps.

Experimental

An overview of the experimental workflow is shown in Figure 1. AAV reference material at known concentrations has been sourced commercially. The stock solution of AAV serotype 5 (AAV5) had a concentration of 2×10^{13} viral particles (VP) per mL and was diluted in 50 mM Na_3PO_4 , 300 mM NaCl. Final sample concentrations were adjusted so that equal injection volumes (50 μL) could be used to generate the calibration curve.

In-house produced samples were expressed in HEK293 cells as published previously.⁷ Following cell harvest, samples were purified as described by the manufacturer using a POROS™ GoPure™ AAVX 1 mL column on an ÄKTA™ avant 150 FPLC system.

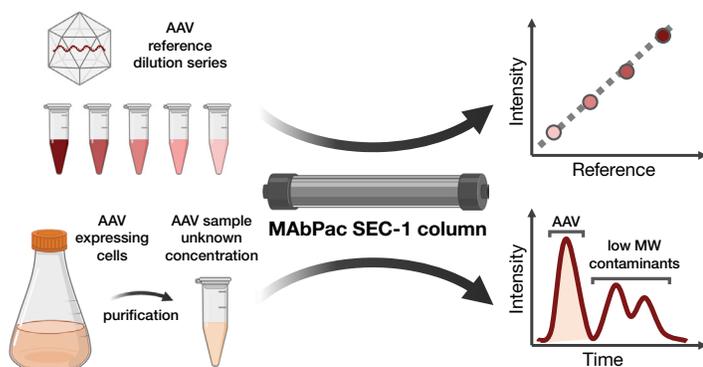


Figure 1. Experimental workflow. AAV reference material was utilized to generate a calibration curve, which was subsequently used to determine AAV titers following affinity purification.

Sample preparation	Reference material was diluted in 50 mM Na_3PO_4 , 300 mM NaCl. In-house produced AAV5 was enriched using one-step affinity purification. All samples were transferred and stored in glass vials.
Chromatography	Thermo Scientific™ Vanquish™ Flex Quaternary Pump, consisting of the following modules: <ul style="list-style-type: none"> • Quaternary Pump F (P/N VF-P20-A) • Split Sampler HT (P/N VH-A10-A) • Column Compartment H (P/N VH-C10-A) • Fluorescence Detector F (P/N VF-D50-A)
Chromatography columns	MAbPac SEC-1 2.1 × 150 mm (P/N 088790)
Chromatography solvents	50 mM sodium phosphate monobasic monohydrate, 300 mM NaCl
Chromatography gradient	Flow 0.075 mL/min, isocratic, column temperature 25 °C
Detector	$\lambda_{\text{ex/em}} = 340/280$ nm
Software	Thermo Scientific™ Chromeleon™ CDS 7.2.10

Results and discussion

Production of recombinant AAVs for the purpose of gene therapy is challenging and requires various analytical assays to monitor PQAs such as product titer. Due to low production yields, it is necessary to develop highly sensitive methods while maintaining good recovery.

We have previously shown the importance of appropriate selection of HPLC vials to obtain consistent and accurate results.⁸ Here, we describe a simple yet precise way of determining AAV product titers even at low sample concentrations.

MAbPac SEC-1 columns efficiently separate lower molecular weight contaminants from AAV capsids. While the corresponding pore size of MAbPac SEC-1 columns would usually be insufficient for the SEC separation of molecules with a molecular weight of that size, here we utilize the inadequate retention to avoid interferences due to remaining contaminants. As shown in Figures 2A and 2B, the AAV peak elutes only 0.2 min later than dextran blue, a common marker used to determine the column void volume indicating no retention. Nevertheless, SmartInject technology integrated in Vanquish UHPLC injection systems eliminates pressure drops derived from the sample loop at atmospheric pressure, thus enabling correct evaluation and

integration of the AAV peak. When injecting 2.5×10^{10} VPs, a signal intensity of approximately 2 mAU was observed indicating the need of higher sensitivity. Therefore, the calibration curve was generated using fluorescence detection (FLD). Doing so, excellent reproducibility was observed as can be seen in Figure 2C.

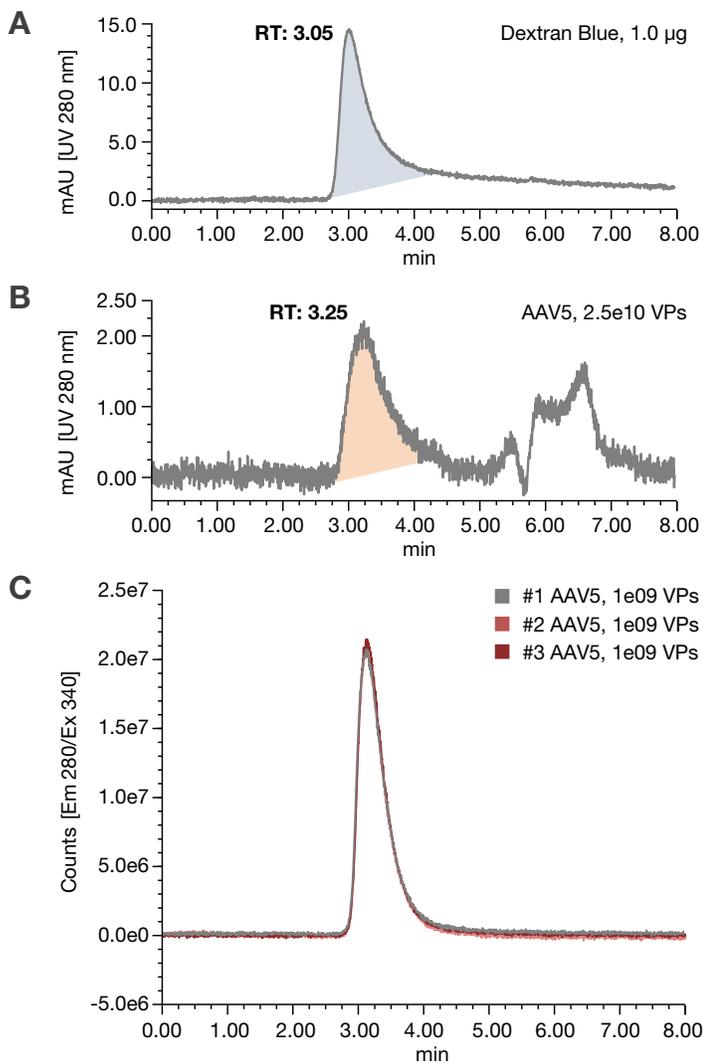


Figure 2. Comparison of retention time (RT) of dextran blue (A) with AAV5 (B) based on UV absorbance at 280 nm; (C) overlay of three chromatograms injecting 1×10^9 viral particles (VPs)

As shown in Figure 3, a calibration curve was generated by injecting 1×10^8 to 2.5×10^{10} VPs on column (50 μ L injections in triplicate). Even at the lowest injection amount, equalling approximately 1 ng of protein, a clear AAV peak was observed highlighting the superiority of FLD. The observed relative standard deviation (RSD) was below 5% and the coefficient of determination (R^2) was determined to be >0.99 . Using the standard deviation and slope of the acquired calibration curve, the limit of detection (LOD) was determined to be at 1.68×10^8 VPs and the limit of quantitation (LOQ) was found to be at 5.10×10^8 VPs.

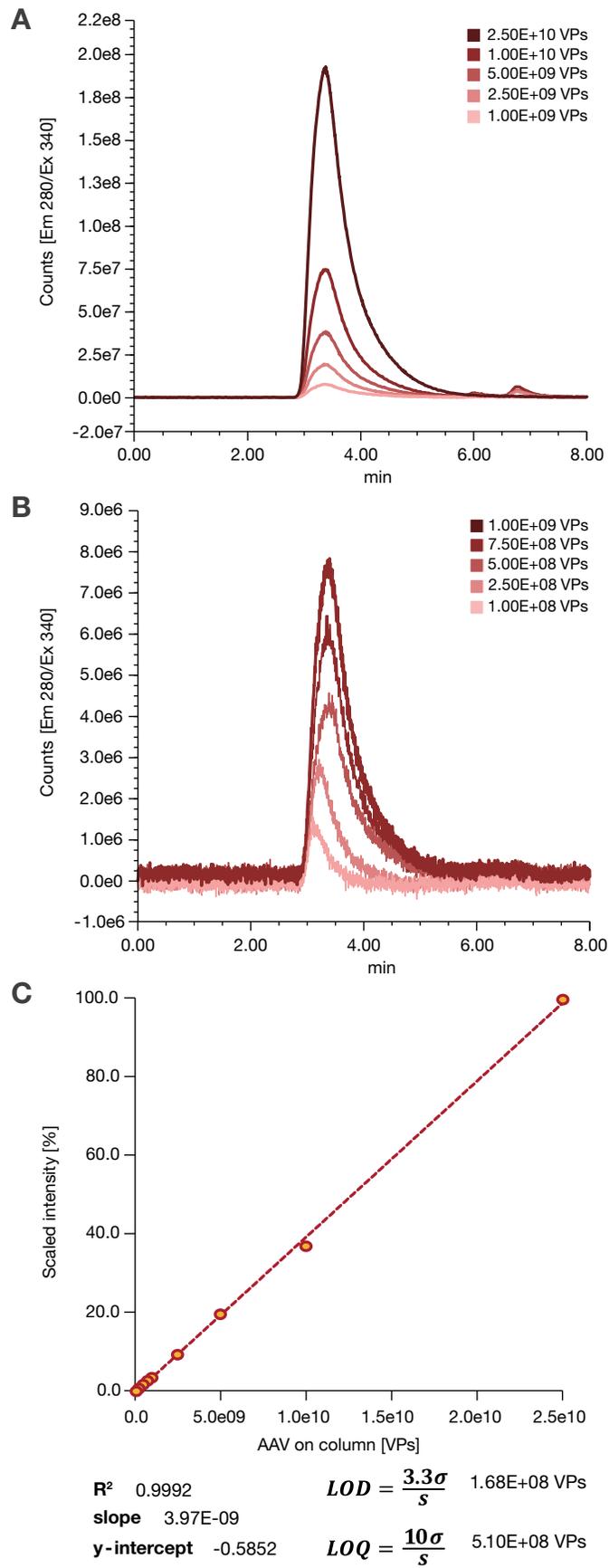


Figure 3. Generation of a calibration curve using a MAbPac SEC-1 2.1×150 mm column. (A) Overlay of acquired chromatograms loading 2.5×10^{10} – 1.0×10^9 VPs and (B) 1.0×10^9 – 1.0×10^8 VPs on column. (C) Calibration curve and result statistics.

Finally, the generated calibration curve was used to determine the titer of a sample with an unknown AAV concentration following upstream processing via affinity purification (Figure 4). Given the known injection amount and the established number of VPs on column, the titer of the sample was determined to be 5.6×10^{11} VP/mL. Additionally, as evidenced by the chromatogram depicted in Figure 4, there was good separation between the observed AAV peak and lower molecular weight (MW) contaminants such as remaining host cell proteins. This indicates that MAbPac SEC-1 columns are suitable for titer analysis of viral vectors as unretained high MW species can be reliably quantitated.

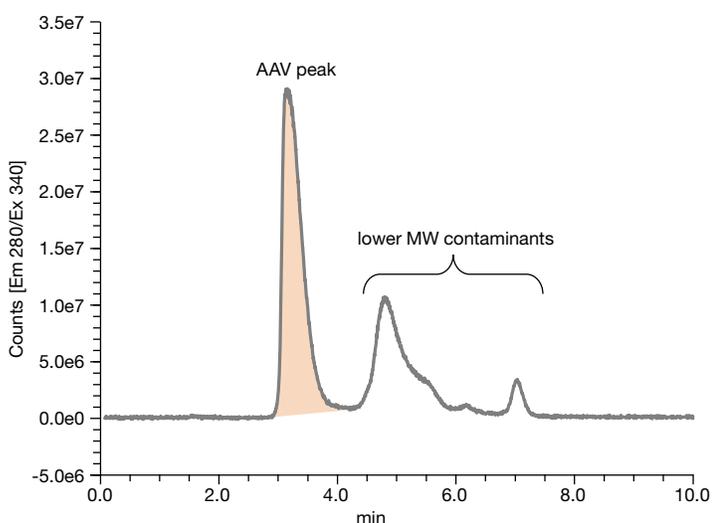


Figure 4. SEC-FLD analysis of an AAV process samples following single-step purification. AAV peak (highlighted in orange) is eluting at a retention time of 3 min, whereas remaining lower molecular weight (MW) contaminants elute after 4.5 min.

Conclusion

In this study, we demonstrated the suitability of SEC using MAbPac SEC-1 columns for titer analysis of viral vector products during downstream processing. While the obtained results are from AAV5, the proposed workflow is also suitable for analysis of other serotypes. The use of FLD yielded in excellent sensitivity with a LOD of 1.68×10^8 VPs and a LOQ of 5.10×10^8 VPs. As discussed previously, the use of low-bind glass vials aided reproducibility with an observed RSD of below 5%. Thus, the presented workflow demonstrates a rapid yet precise and reproducible way of establishing AAV titers during recombinant AAV manufacturing.

References

1. Au, H. K. E.; Isalan, M.; Mielcarek, M. Gene therapy advances: a meta-analysis of AAV usage in clinical settings. *Front Med. (Lausanne)* **2021**, *8*, 809118, doi:10.3389/fmed.2021.809118.
2. Gimpel, A. L. 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, doi:10.1016/j.omtm.2021.02.010.
3. Srivastava, A.; Mallela, K. M. G.; Deorkar, N.; Brophy, G. Manufacturing challenges and rational formulation development for AAV viral vectors. *J. Pharm. Sci.* **2021**, *110*, 2609–2624, doi:10.1016/j.xphs.2021.03.024.
4. Wang, D.; Tai, P. W. L.; Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* **2019**, *18*, 358–378, doi:10.1038/s41573-019-0012-9.
5. Mittereder, N.; March, K. L.; Trapnell, B. C. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* **1995**, *70*, 7498–7509, doi:10.1128/JVI.70.11.7498-7509.1996.
6. McIntosh, N. L. et al. Comprehensive characterization and quantification of adeno associated vectors by size exclusion chromatography and multi angle light scattering. *Sci. Rep.* **2021**, *11*, 3012, doi:10.1038/s41598-021-82599-1.
7. Strasser, L. et al. Proteomic landscape of adeno-associated virus (AAV)–producing HEK293 cells. *Int. J. Mol. Sci.* **2021**, *22*, 11499.
8. Thermo Scientific Application Brief 7417–Choosing the right sample vial for analysis of AAV to prevent sample loss, **2021**. <https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/ab-74172-lc-choosing-vials-aav-analysis-ab74172-en.pdf>

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