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Establishing a Small Scale AAV Empty/Full Separation Process using Sartobind® Q Lab

Frederik Meierrieks[1], Benjamin Graf[1], John S Cashman[2]
[1] Sartorius Lab Instruments GmbH & Go, KG, Otto-Brenner-Strasse 20, 37079 Goettingen, Germany
[2] Sartorius UK Limited, Longmead Business Centre, Belnheim Road, Epsom, KT19 9QQ, UK

Correspondence
Email: john.cashman@sartorius.com

Abstract

Adeno-associated viruses (AAVs) are small, non-enveloped viruses with a diameter of 20-25 nm that have gained interest as delivery vehicles for gene therapies. Their low pathogenicity and ability to transduce many different cell types makes them ideal candidates for the treatment of a broad range of diseases. Even though the number of approved AAV-based treatments is continually increasing, AAV production and purification remains challenging. During production in cell culture, AAV particles that harbor ssDNA (full particles) encoding a therapeutic transgene as well as particles without any nucleic acid cargo (empty particles) are produced. Therefore, enrichment of full particles is a key requirement in downstream processing. In this study, we set out to describe how a serotype-independent empty/full separation process can be established, using Sartobind® Lab anion exchange (AEX) chromatography units. Once optimized, the final method can be applied without a liquid chromatography (LC) system, using a syringe and appropriate buffers.

Introduction

To develop our strategy for optimizing empty/full AAV separation, AAV particles with a ssDNA transgene were produced in HEK293 suspension cultures . The cells were lysed 72 hours post-transfection to ensure a maximum yield of produced AAV particles. However, the separation method we describe is independent of the production process and can therefore be applied to AAV particles produced by other methods described in the published literature, such as in adherent cell cultures or *Sf* 9 insect cell cultures.

Clarification of the crude cell culture is required before chromatography. Even though centrifugation followed by microfiltration is widely used for this purpose, a membrane-only based clarification is faster, easier and does not require expensive equipment. Using a combination of a microfiltration membrane with a filter aid material like diatomaceous earth (DE) is an effective method to prevent filter blocking. To guarantee the best filtration performance while maintaining a high recovery of AAV particles in the filtrate, Meierrieks *et al.* used a design of experiments (DoE) approach to optimize the DE-supported clarification process. For standard HEK293 suspension cell cultures, adding approximately half the wet-cell weight of DE to the culture medium prior to filtration using a 0.22 µm polyethersulfone (PES) filter was recommended [1].

Different methods exist to purify AAV particles from clarified cell cultures. In basic research, a combination of PEG precipitation with density gradient ultracentrifugation (DGUC) is widely used. As this method is time-consuming and has limited scalability, chromatographic solutions have been developed. Both affinity chromatography (AC) and cation-exchange chromatography (CEX) can be used to capture AAV particles prior to empty/full separation. While AC can offer high selectivity for AAV particles, purification speed represents a major drawback. To date, all commercially available AAV affinity matrices are resin-based, requiring limited flow rates, especially during sample loading, and exhibiting size exclusion effects. Monolithic CEX can be used to overcome these limitations. Monoliths are convective matrices that allow a flow-independent interaction between the particles of interest with the stationary phase ligands. Rieser *et al.* have described and compared the different chromatographic methods for purifying AAV particles [2]. A further benefit of CEX-based AAV purification is the possibility for high throughput optimization of the purification process, using monolithic deep-well plates [3].

As for other steps in the downstream processing of AAV samples, different methods can be used to enrich full particles while removing empty particles. DGUC is a well-established process in lab-scale workflows, which can simultaneously isolate AAV from a clarified cell culture and separate the empty from full particles. However, as mentioned above, this process has limited scalability and requires access to an ultracentrifuge. Therefore, scalable chromatographic methods have been developed. Besides the use of resin-based ion exchanged matrices, monolithic columns are widely used. As mentioned before, monoliths are convective matrices that are not limited by diffusion, so a flow rate independent separation of particles like AAVs is possible [4,5]. Membrane adsorbers may be a useful equivalent to monoliths, as mass transport is also mainly driven by convective flow in these matrices [6].

In this protocol, we have established a method for empty/full separation of AAV serotype 8 (AAV8), using membrane adsorbers. We have also shown that our method can be easily transferred to other AAV serotypes, including AAV2 and AAV5. Once optimized, the method can be used for equipment-free empty/full separation, using an AEX membrane adsorber unit in combination with standard syringes and appropriate buffers.

Materials

Buffers

Buffer Name	Composition
NaCl Loading Buffer	20 mM Bis-Tris propane pH 9.0
NaCl Elution Buffer	20 mM Bis-Tris propane pH 9.0, 1 M NaCl
$MgCl_2$ Loading Buffer	50 mM Tris pH 8.5, 2 mM $MgCl_2$
$MgCl_2$ Elution Buffer	50 mM Tris pH 8.5, 50 mM $MgCl_2$

Table 1. *Overview of the buffer systems used for scouting and preparative empty/full separations.*

Laboratory Equipment and Consumables

Product Name	Order Number | Supplier
Liquid chromatography system	Cytiva
Omnifix® Luer-lock syringes	B Braun
Sartobind® Q Lab, 0.41 mL MV	93IEXQ42GB-12--A
Octet® R8 BLI System	Sartorius https://sar.to/e0dfg
Octet® AAVX Biosensors	18-5160
AAV Reference Standards	Progen Biotechnik
QIAcuity dPCR System	Qiagen

Table 2. *Overview of the laboratory equipment and consumables required for empty/full separation and BLI- and dPCR-based analysis of eluent fractions.*

Methods and Results

AAV Capsid Titer Determination

The capsid titer of AAV-containing fractions was determined using a Biolayer Interferometry (BLI) system equipped with AAV affinity biosensors (Sartorius, Germany). Quantitation was performed at 30°C with a shake speed of 1000 rpm and a total read time of 900 s using the pre-defined AAV quantitation program. As reference standard material for the BLI measurements, AAV particles for each of the serotypes tested were purchased from Progen Biotechnik (Heidelberg, Germany).

ELISA kits are also commercially available and can be used as an alternative method for AAV capsid titer determination. However, these kits are serotype-specific and the ELISA measurements only offer a narrow linear range for accurate titer determination, whereas an Octet® BLI system in combination with AAVX biosensors allows measurements for multiple serotypes between ~8.5 x 10^8 and 1.0 x 10^{12} vp/mL [7,8].

AAV Genome Titre Determination

AAV genome titers (vg) were quantified by digital polymerase chain reaction (dPCR), using the QIAcuity One (Qiagen, Germany) system with 24 well nanoplates (Qiagen, Germany). Samples underwent serial dilution in dPCR buffer, consisting of TE buffer (Thermo Fisher Scientific, USA), 0.01% (v/v) Pluronic F 68 (Sigma Aldrich, Germany), and 100 mg/mL Poly A Carrier RNA (Roche, Switzerland). The diluted samples were incubated at 95 °C for 30 minutes. In a following step the dPCR measurements were performed using the QIAcuity Probe Mastermix solution (Qiagen, Germany) supplemented with forward and reverse primers (final concentration of 800 nM) and a specific probe (Integrated DNA Technologies, USA) targeting the SV40 polyadenylation signal (final concentration of 400 nM).

Empty/Full Separation Process Development

To establish an empty/full separation using the AEX membrane adsorber, approximately 1.0 x 10^{13} affinity purified AAV particles were loaded onto a membrane adsorber unit, equilibrated either in NaCl or $MgCl_2$ Loading Buffer. Prior to sample loading, the affinity purified material was diluted 1:20 with the respective buffer system used for equilibration. All further purification steps were performed using a liquid chromatography system (LC) at a flow rate of 5 MV/min (2 mL/min). After loading, a washing step was applied, using 15 MV of the respective Loading Buffer. Different elution methods in terms of ionic strength and gradient profiles were tested and are described in detail below.

For all LC system-driven processes flowthrough and eluent absorbance was measured at 280 nm and 260 nm to allow identification of AAV and differentiation between empty and full particles based on A_{280}/A_{260} ratios.

E/F Separation using Linear Elution Gradients

Monovalent salt elution (NaCl): Using the monovalent salt buffer system, a linear gradient from 0-20% buffer B was applied over 4 min at a flow rate of 5 MV/min (2 mL/min, Buffer A: NaCl Loading Buffer; Buffer B: NaCl Elution Buffer). Fractions of 1 mL were collected and stored at 4 °C until further analysis.

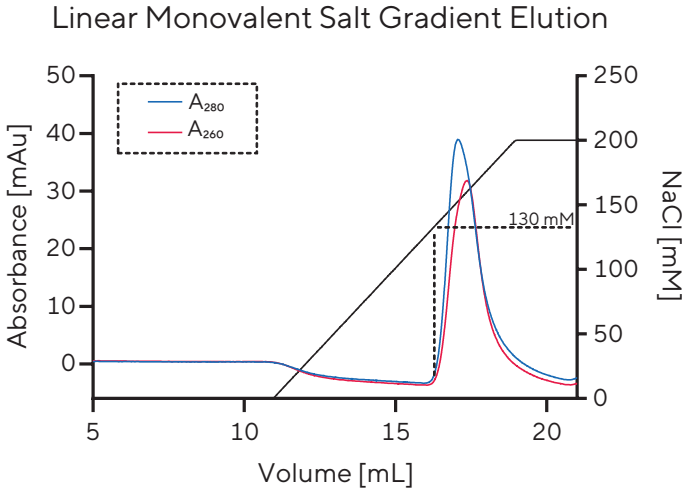

Figure 1. *Chromatogram of a purification cycle used to test empty/full separation of purified AAV8 particles with a monovalent salt (NaCl) buffer system. A linear elution gradient of increasing ionic strength was applied (black) leading to a single peak eluting between 15 and 20 mL and reaching an absorbance maxima of ~40 mAU. Absorbance was monitored at 280 nm (blue) and 260 nm (red).*

The chromatogram corresponding to a linear NaCl elution gradient with AAV8 particles (Figure 1) showed a single peak eluting between 15 and 20 mL. Elution started at an ionic strength of ~130 mM NaCl. However, no effective separation between empty and full AAV8 particles was detected, the slight shift between A_{280} and A_{260} ratios within the single elution peak confirmed a mixture of empty and full particles.

Divalent salt elution ($MgCl_2$): For elution using the divalent salt buffer system, a linear gradient from 0-100% buffer B over 4 min at a flow rate of 5 MV/min (2 mL/min) was applied (Buffer A: $MgCl_2$ Loading Buffer; Buffer B: $MgCl_2$ Elution Buffer). Fractions of 1 mL were collected and stored at 4 °C until analysis.

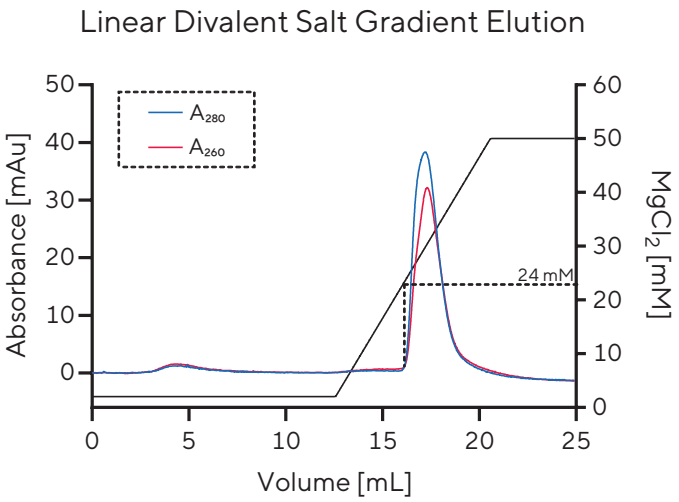

Figure 2. *Chromatogram of a purification cycle used to test E/F separation of AAV8 particles with a divalent salt ($MgCl_2$) buffer system. A linear elution gradient of increasing ionic strength was applied (black) leading to elution in a single peak with an absorbance maxima of ~40 mAU, between 15 and 20 mL. Absorbance was monitored at 280 nm (blue) and 260 nm (red). leading to a single peak eluting between 15 and 20 mL and reaching an absorbance maxima of ~40 mAU. Absorbance was monitored at 280 nm (blue) and 260 nm (red).*

The chromatogram corresponding to a linear $MgCl_2$ gradient with AAV8 particles (Figure 2) showed a single peak eluting between 15 and 20 mL, corresponding to an ionic strength of ~24 mM $MgCl_2$. This was comparable to the linear NaCl elution (see Figure 1), with the empty and full AAV8 particles remaining separated. The slight offset between A_{260} and A_{280} indicated that a mixture of empty and full AAV8 particles was marginally separating within the single elution peak. Therefore, step gradients were chosen to optimize the E/F separation.

E/F Separation Using Multi-Step Elution Gradients

Monovalent salt elution (NaCl):

Based on the results of the linear elution gradient using the NaCl buffer system (Figure 1), a multi-step elution gradient was applied, with an initial step to 80 mM NaCl. Further steps, each corresponding to a 20 mM increase in NaCl concentration, were applied to effect separation between empty and full AAV8 particles (Figure 3A). Each increase in NaCl concentration was applied as a short (2 seconds) linear gradient. Eluates were collected in 1 mL fractions for analysis using a combination of BLI capsid titer determination and dPCR measurements for viral genome titer determination. The analytical measurements revealed a successful empty/full separation. The separation process was further optimized to use an initial step to 120 mM NaCl followed by further steps in 10 mM NaCl increments (Figure 3B). This allowed the enrichment of up to 73.2% genome-filled AAV8 particles in the second elution peak (Figure 3B), corresponding to an enrichment factor of ~7.8-fold over the affinity purified AAV8 (load).

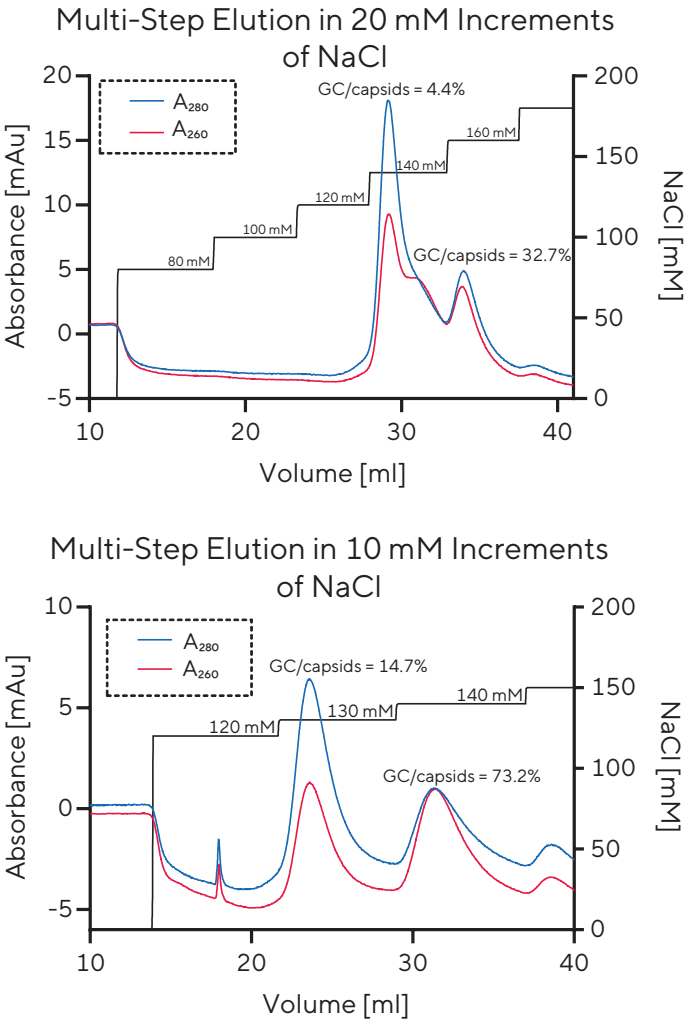

Figure 3. *Chromatograms of empty/full separation using the monovalent salt (NaCl) buffer system and stepwise elution. A. The chromatogram shows two distinct peaks appearing between 28 and 36 mL. The first elution peak reaches an absorbance maxima of 19 mAU at a volume of ~29.5 mL. The second elution peak at 34 mL reaches a maximum absorbance of 5 mAU. Analytical measurements (not shown) confirmed an E/F ratio of 4.4% for the first peak and 32.7% for the second peak. B. The chromatogram shows three distinct peaks between 20 and 40 mL. The first elution peak reaches an absorbance maxima of 7.5 mAU at 24 mL and had an E/F ratio of 14.7%. The second elution peak at 32 mL with a significantly lower absorbance showed an E/F ration of 73.2%. The third peak at 39 mL was not analyzed due to its low absorbance signal. Each chromatogram shows absorbance at 280 nm (blue) and 260 nm (red), and the NaCl concentration (black).*

Divalent salt elution ($MgCl_2$):
For a multi-step elution gradient using the divalent salt buffer system, a first isocratic elution step to 22.5 mM $MgCl_2$ was selected, based on the outcome of the linear elution experiment (Figure 2). Subsequent elution steps were applied as short (2 s) linear gradients in increments of 2.5 mM $MgCl_2$. During sample loading at an ionic strength corresponding to 2.0 mM $MgCl_2$ a first peak containing no filled AAV8 particles appeared. The increasing $MgCl_2$ concentration led to the formation of two main elution peaks. The first elution peak appeared at a $MgCl_2$ concentration of 25 mM and showed a leading shoulder. This first elution peak contained 1.3 % full AAV8 particles, and was followed by a second elution peak containing 63.9 % of the full AAV8 particles eluting at 27.5 mM $MgCl_2$. This corresponds to an enrichment factor of 6.9-fold relative to the affinity purified AAV8 sample (Figure 4).

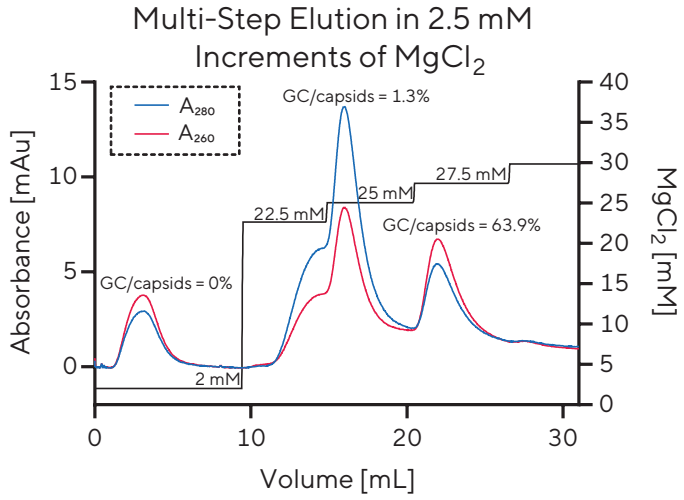

Figure 4. *Chromatogram showing empty/full separation of AAV8 particles using stepwise elution with a divalent salt ($MgCl_2$) buffer system. The chromatogram shows three distinct elution peaks. The first peak elutes 1 to 6 mL after loading the affinity purified AAV8 material. Analytical measurements (not shown) confirmed that this peak did not contain any AAV particles (0% GC/capsids). A second elution peak at 16 mL post-loading (25 mM $MgCl_2$) reached an absorbance maxima of 14.5 mAU and contained 1.3% full particles. The third elution peak at 22 mL post-loading (27.5 mM $MgCl_2$) reached a maximum absorbance of 7 mAU and contained 63.9% filled capsids. The chromatogram shows absorbance at 280 nm (blue) and 260 nm (red), and the $MgCl_2$ concentration (black).*

E/F Separation using a Flowthrough Approach
To establish a flowthrough strategy for more efficient separation of empty and full AAV particles using the divalent salt buffer system, a well-defined proportion of buffer B was used to equilibrate the AEX membrane adsorber unit before sample loading. The goal was to prevent binding of empty AAV particles to the stationary phase while full particles are captured by the positively charged (cationic) ligand.

Based on the results from stepwise elution using the $MgCl_2$ buffer system (Figure 4) affinity purified AAV8 was adjusted to a final $MgCl_2$ concentration of 24.5 mM before loading onto an AEX membrane adsorber unit equilibrated with the same $MgCl_2$ concentration. Figure 5A shows the chromatogram from this separation strategy, where the load peak contained only 1.9% full AAV8 particles. With a subsequent increase in $MgCl_2$ concentration to 31 mM, an elution peak containing 44.9% full AAV8 particles was observed. This corresponds to a 4.83-fold enrichment factor relative to the load.

To confirm that these separation strategies could also be applied to other AAV serotypes, we used the same approach described in the previous sections for E/F separation of AAV2 and AAV5. Figure 5B shows the optimized flowthrough E/F separation strategy with the divalent salt buffer system for AAV5 particles. The loading step for AAV5 was performed at an ionic strength corresponding to 29 mM $MgCl_2$, with a resulting peak containing 4.9% full particles. Elution at 33 mM $MgCl_2$ produced a major peak containing 71.0% full AAV5 particles. This corresponds to an enrichment factor of ~7-fold compared to the affinity purified starting material. Figure 5C shows the chromatogram of a fully established E/F flowthrough separation for AAV2. During sample loading at 15.5 mM $MgCl_2$, the flowthrough peak contained 0.4% full particles, and subsequent elution with 20.25 mM $MgCl_2$ resulted in a peak containing 23.8% full AAV2 particles. Compared to the affinity purified AAV2 material, the flowthrough method led to a 4-fold enrichment factor.

To enable rapid empty/full separation of AAV particles without a liquid chromatography system, the flowthrough separation method was adapted to use loading and elution buffers that were formulated to the previously determined $MgCl_2$ concentrations for each AAV serotype. For this separation method, the LC system was substituted by a standard Luer-lock syringe, connected directly to the inlet of a Sartobind® Q Lab membrane adsorber unit (Figure 5D). Fractions were collected in tubes placed under the outlet of the membrane adsorber unit. The syringe application allowed a 5.35-fold enrichment of full AAV8 particles (49.8% full particles) compared to the load (9.3% full particles, Figure 5E).

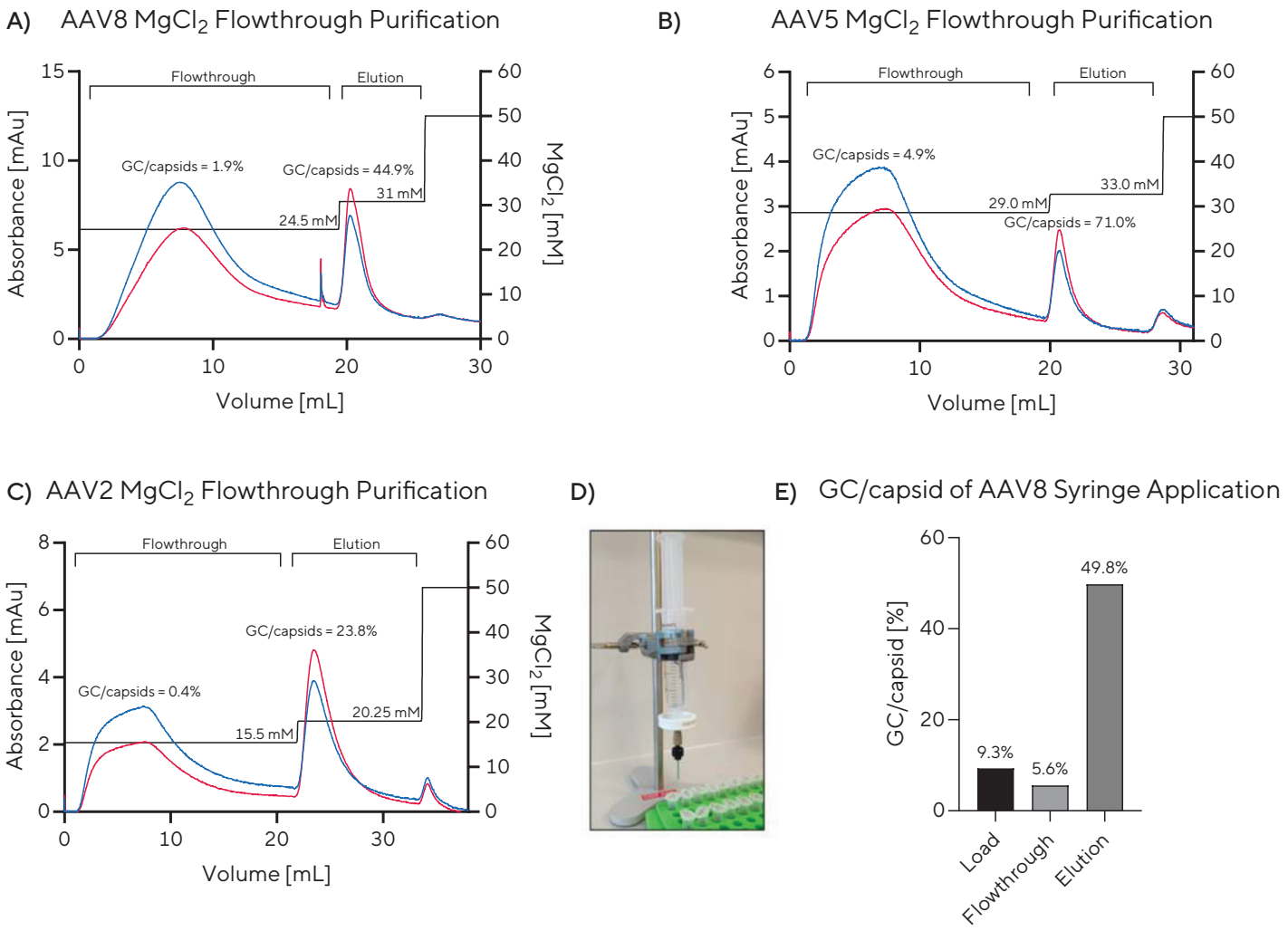

Figure 5. *Results of the flowthrough strategy for empty/full separation of different AAV serotypes. A-C. Elution profiles for AAV8, AAV5 and AAV2, respectively, showed enrichment of full AAV particles in the eluate fractions. D. The setup used for flowthrough empty/full separation without a LC system, showing a buffer-filled syringe connected to an AEX membrane adsorber unit and fraction collection tubes underneath. E. Capsid genome content in the affinity purified sample (load), and the peaks following empty/full separation (flowthrough, elution) for AAV8, showing fewer filled particles in the flowthrough.*

Summary

In this study, we have shown that Sartobind® Q Lab membrane adsorber units can be used to separate empty from full AAV particles. Following our process development approach for AAV8, we found that the same approach could also be applied to optimize empty/full separation for other serotypes like AAV5 and AAV2 . Apart from the three tested AAV serotypes it can be assumed that the described scouting strategy can also be transferred to all other AAV serotypes. Effective empty/full separation for different AAV subtypes could also be achieved using both monovalent (e.g., NaCl) and divalent (e.g., $MgCl_2$) salt-based buffer systems. The scouting and optimization approach we have developed is summarized in Figure 6.

Our final strategy allows for separation of empty and full AAV particles in a manner where the empty particles do not bind to the stationary phase while full particles do. Full particles are subsequently desorbed from the stationary phase with a single elution step, using a buffer with increased ionic strength. Since this strategy only requires two steps (loading and elution) it can be easily applied using a syringe, avoiding the need for time-consuming setup and access to a liquid chromatography system, especially when handling a small number of samples in standardized processes.

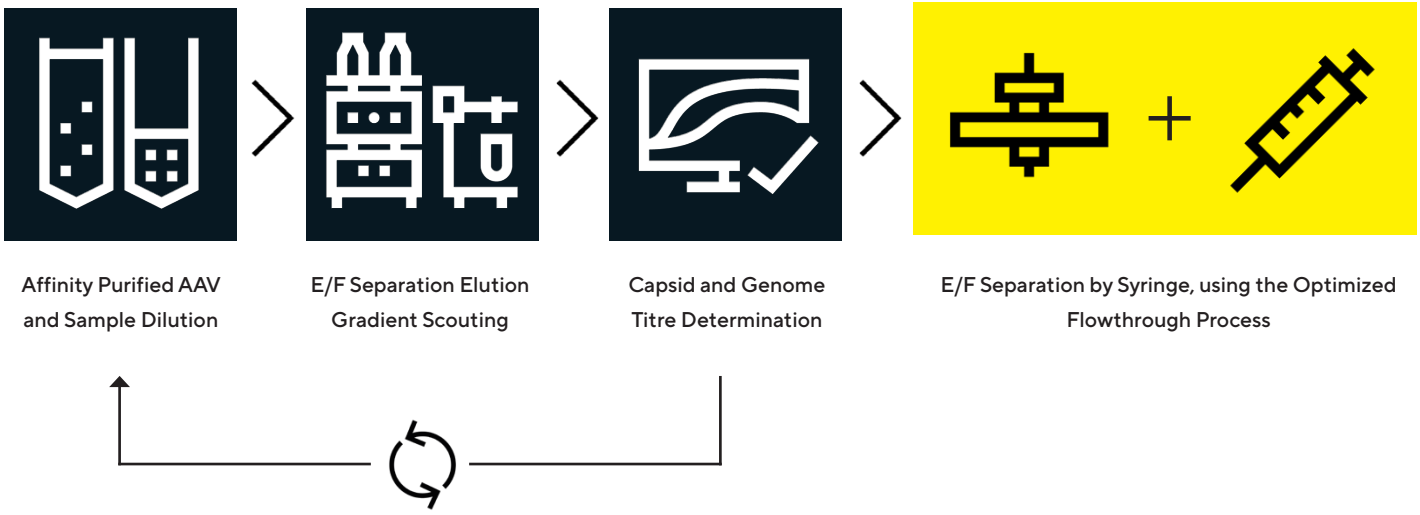

Figure 6. *Overview of the process development steps used to optimize separation of empty from full AAV particles using an anion exchange membrane adsorber. Syringe-driven purification (yellow background) can be easily applied with the optimized flowthrough strategy (Figure 5), as a simple benchtop alternative to using a LC system.*

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North America
Sartorius Corporation
300 West Morgan Road
Ann Arbor, Michigan 48108
USA
Phone +1 734 769 1600
Email: orders.US07@sartorius.com

Europe
Sartorius UK Ltd.
Longmead Business Centre
Blenheim Road
Epsom
Surrey, KT19 9QQ
United Kingdom
Phone +44 1763 227400
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202
Email: orders.US07@sartorius.com

For further information, visit
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