Comparing CsCl Density Gradient Ultracentrifugation and Anion Exchange Chromatography for Full Capsid Enrichment of Novel Adeno-Associated Viral Vectors

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INTRODUCTION

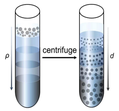
A key challenge in the manufacturing of recombinant Adeno-Associated Viruses (rAAV) is the removal of capsids which do not contain the complete transgene of interest. It is common for triple transfection processes using Human Embryonic Kidney (HEK) 293 cells to produce more empty capsids than full. Since empty capsids cannot transduce target cells in a patient, their presence can lower the efficacy of an rAAV gene therapy. Partially packaged capsids can also be found in rAAV preparations and should be tracked across enrichment steps. There are two predominant operations currently used by the industry to remove empty capsids during rAAV manufacturing:

- Cesium chloride (CsCl) density gradient ultracentrifugation separates empty and full particles by mass. This process is agnostic to capsid structure and can therefore separate full rAAV from empty rAAV using standard protocols. The major drawbacks to this method are long process times, lack of scalability, manual product collection, and low step yields.
- Anion Exchange (AEX) chromatography exploits slight surface charge differences between full and empty capsids to achieve separation in a bind-and-elute mode. Monolith technology has emerged as an attractive stationary phase for viral vector purification due to large porosity of flow channels and convective mass transfer properties.

Here, we present data for both ultracentrifugation and chromatographic separation methods for rAAV polishing, and compare performance in terms of full capsid enrichment, process yield, residual impurity clearance, and process time. Additionally, a review of data generated for multiple capsids and transgene constructs offers interesting insights to the relative influence of capsid and transgene properties on AEX purification.

OVERVIEW OF CAPSID ENRICHMENT TECHNIQUES

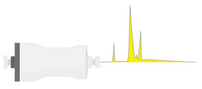
Cesium Chloride (CsCl) Ultracentrifugation



Principle of Operation

- A gradient solution of CsCl allows particles to separate by their respective densities.
- Source material is pipetted into tube and spun in ultracentrifuge for up to 24 hours.
- Density difference between full and empty AAV capsids result in separation.
- Product material is collected manually via needle and syringe.

Anion Exchange (AEX) Monolith Chromatography



Principle of Operation

- Affinity purified AAV is bound to AEX monolith at low conductivity and alkaline pH.
- An increasing linear conductivity gradient is applied to the column.
- Surface charge difference between full and empty AAV capsids result separation.
- Product peak can be collected using automated parameters
- Monolith chromatography features a network of flow channels within singular structure. Binding sites are in the convective path, enabling short residence times.

MATERIALS & METHODS

Source Material:

- Capsid and transgene combinations were produced using a triple transfection HEK293 suspension expression system (Table 1). Both CapA and CapB are AAV9 derived capsids from the TRACER™ Platform.
- Each transgene is a proprietary sequence with a self-complementary structure.

Ultracentrifugation:

- Ultracentrifugation conditions were first purified by ion exchange polishing chromatography operated in product flowthrough mode to help clear impurities (but not empty capsids).

AEX Monolith:

- All AEX Monolith purifications used CIMmultus QA (2 µm pore) monoliths from Sartorius BIA Separations.
- The same AEX enrichment method and linear salt gradient elution strategy was used in all experiments.

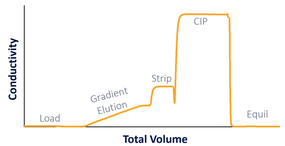
Analytics:

- Either SEC-MALS or AUC was used to assess full capsid content in purified preparations. Data on partially full capsids is provided for samples analyzed by AUC. Vector genome (VG) yield was measured by ddPCR assay.

Table 1. Capsids and transgenes evaluated.

Capsid	Transgene
CapA	Tg1
CapB	Tg2
	Tg3

Figure 1. Conductivity trend during monolith experiments.



RESULTS & DISCUSSION

AEX Monolith and CsCl Ultracentrifugation for two novel AAV constructs

CapA:Tg1

Figure 2. AEX monolith purification of CapA:Tg1 at different scales.

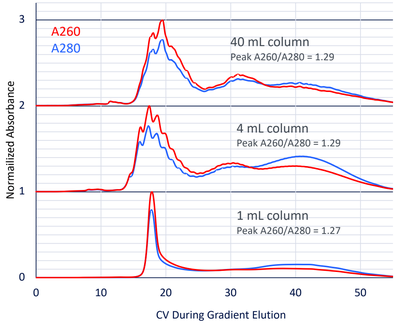


Table 2. Product quality & yields for CapA:Tg1 prepared by AEX and CsCl.

	AEX Monolith			CsCl Ultracentr.
	1 mL CV	4 mL CV	40 mL CV	
% Full	74% full (SEC-MALS)	78% full, 7.7% partial (AUC)	89% full, 0% partial (AUC)	96% full (SEC-MALS)
VG Yield	57%	53%	40%	31%
HMW (SEC)	0.2%	0.2%	0.1%	0.1%
HEK HCP (ng/1E+13 vg)	BLQ	10.6	BLQ	BLQ
Free hcDNA (ng/1E+13 vg)	BLQ	BLQ	BLQ	BLQ
Nuclease Resistant DNA (ng/1E+13 vg)	472 *	N/A	N/A	937 *
Process time	2 hour per cycle	2 hour per cycle	2 hour per cycle	24 hour per spin

** Processed from same lot of source material*

CapA:Tg2

Figure 3. Separation of full and empty capsids in distinct bands following CsCl ultracentrifugation.

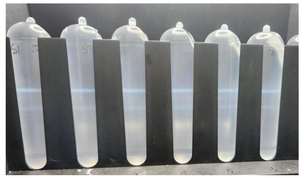


Table 3. Product quality & yields for CapA:Tg2 prepared by AEX and CsCl. The same lot of source material was used for both purification methods.

	AEX Monolith (1 mL CV)	CsCl Ultracentr.
%Full (SEC-MALS)	67% **	84%
VG Yield	58%	40%
HCP (ng/1e13 vg)	<LOQ	1059
Free hcDNA (ng/1e13 vg)	20.38	<LOQ
Nuclease Resistant DNA (ng/1e13 vg)	292	613

*** Adjustment to peak collection strategy expected to provide ≥ 70% full with ≥ 50% yield*

- Both CapA:Tg1 and CapA:Tg2 can be enriched to ≥70% full capsid using either AEX monolith or CsCl ultracentrifugation.
- AEX monolith offered consistent performance scaling up from 1 mL to 40 mL CV for CapA:Tg1.
- Both strategies offer similar levels of residual HCP clearance. AEX monolith may offer slightly improved clearance of nuclease resistant DNA.

	AEX Monolith	CsCl Ultracentrifugation
Benefits	• Linear scale-up. • Short process time. • Higher yield. • Opportunity for adventitious viral clearance.	• Robust separation with high purity. • Serotype agnostic – minimal optimization needed to prepare enriched full capsid material.
Challenges	• Serotype dependent – need to optimize construct-specific elution and peak collection strategy. • May be difficult to sufficiently enrich feeds with very low full capsid content (< 10%).	• Scale-out, rather than scale-up. • Long process time. • Manual product collection. • Lower yield. • Other unit ops need to provide viral clearance.

Impact of transgene variation on AEX monolith enrichment

Figure 4. AEX monolith purification of CapA packaged with Tg1, Tg2, and Tg3 (1 mL column volume scale).

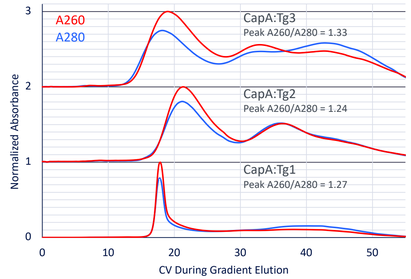
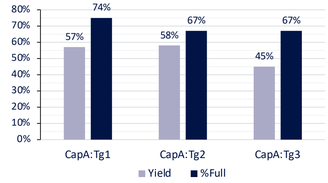


Figure 5. AEX monolith full capsid enrichment (SEC-MALS) and step yields for CapA packaged with Tg1, Tg2, and Tg3.



- Similar monolith performance with same capsid and three different transgenes. All product peak A260/A280 ratios > 1.2.
- Post-product elution peaks differ with transgene, possibly due to varying levels of partial capsid impurities.
- 70 ± 5% full and 50 ± 5% yield for all three transgene constructs tested.

Impact of capsid variation on AEX monolith enrichment

Figure 6. AEX monolith purification of CapA & CapB packaged with Tg1 (1mL column volume scale).

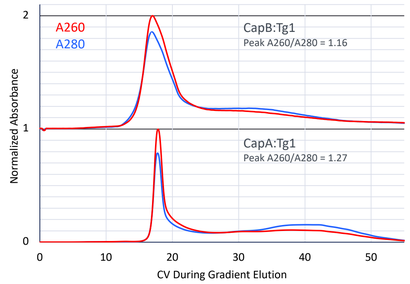
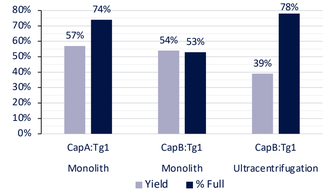


Figure 7. AEX monolith full capsid enrichment (SEC-MALS) and step yields for CapA & CapB packaged with Tg1.



- CapB:Tg1 is challenging to purify by AEX monolith: A260/A280 < 1.2 and product pool < 60% full. Further development work needed.
- Ultracentrifugation enriches CapB:Tg1 to 78% full with 39% yield.
- Results suggest AEX monolith is more sensitive to changes in capsid properties than changes in packaged transgene.

CONCLUSIONS

- **AEX Monolith chromatography is a scalable method to enrich rAAV capsids to ≥ 70% full.** AEX monolith provided:
  - Higher yields than CsCl ultracentrifugation with shorter run times (a few hours as opposed to 24+ hours)
  - Similar levels of residual host cell protein and DNA reduction.
  - Repeatable performance with automated product collection rather than manual operations.
- **Since AEX monolith may require more development than ultracentrifugation, it is ideal for clinical stage products.**
  - For a well-developed monolith process, minimal additional optimization may be needed when the same capsid is packaged with a different transgene.
  - Changes to the novel capsid structure will likely require dedicated monolith development efforts, even if serotype properties are maintained.
- **Ultracentrifugation works well across all constructs and therefore is useful for discovery and pre-clinical production.**
  - The phase in the product's lifecycle determines the most appropriate enrichment method.

ACKNOWLEDGEMENTS

- Voyager Therapeutics Pilot Team for generating large scale monolith and ultracentrifugation data.
- Voyager Therapeutics Process Analytics Team for execution of ddPCR, SEC-MALS, AUC, and residual host cell impurity assays.
- Sartorius BIA Separations for consultation on monolith operating parameters.