Simplifying Progress

New Empty|Full Methods to Enable Robust Manufacturing of AAV Products

October 2023

SARTORIUS

About Sartorius BIA Separations

- The **leading developer** of **monolith technology** and the exclusive producer of CIM® (Convective Interaction Media) monolithic chromatographic columns **celebrating the 25th anniversary** just today.
- A specialist in the purification of large biological molecules and viral particles for **gene therapy and the vaccine markets.**
- **Sartorius center of excellence in gene therapy** offers solutions for downstream process development and manufacturing and for analytical methods applicable to multiple large molecules, e.g. AAV, Adeno, Flu, pDNA, mRNA.
- **Supplies unique monolithic chromatographic columns** complimentary to porous particles and membranes.

Notable years:

Incorporated	1998
Relocated	2011
Acquired	2020

Sartorius BIA Separations Product and Solutions Portfolio

BIA's Proprietary Product and Services Offering

CIMmultus® Monolith production-scale columns

- **10 to 100 times** higher capacity
- **Up 3 times** higher recovery
- **Up to 3 times** lower drug manufacturing cost
- Column sizes **40L or more**

CIMac™ monolith process control columns

- **Higher integrity** information at **lower cost** and **better accuracy**
- **Biocompatible**

PATfix® solutions

- Achieves rapid, **high-resolution** separations in minutes
- **Fast** and **reproducible** HPLC monitoring of **large** biomolecules

Process and method development services

- The **leading expert** on large biomolecules and viral particles
- Drives **long-term, embedded** customer relationships
- **Tailored services** for each clinical phase up to and including production
- **More than 50** process and method development experts

World-class team

State of the art facilities in Slovenia

Experts in Express Bioproduct Manufacturing Process Development – No Royalties

- pDNA including Corona, purity is the key for better transfection and purer mRNA
- Minicircle DNA (shorten the pDNA)
- ssRNA and dsRNA, platform process from E.coli to mRNA including Corona
- Adeno virus, more than 20 years experience, including Corona
- AAV (all serotypes, > 20 tested)
- Life influenza virus (all serotypes)
- Vaccinia/MVA
- Exosome
- Bacteriophage
- VLPs and inactivated vaccines (including Flu and Corona)
- IVIG
- IgM and many more

>50 pDNA, mRNA, virus DSP cGMP processes tech transferred to CMOs, sponsors, including Corona.

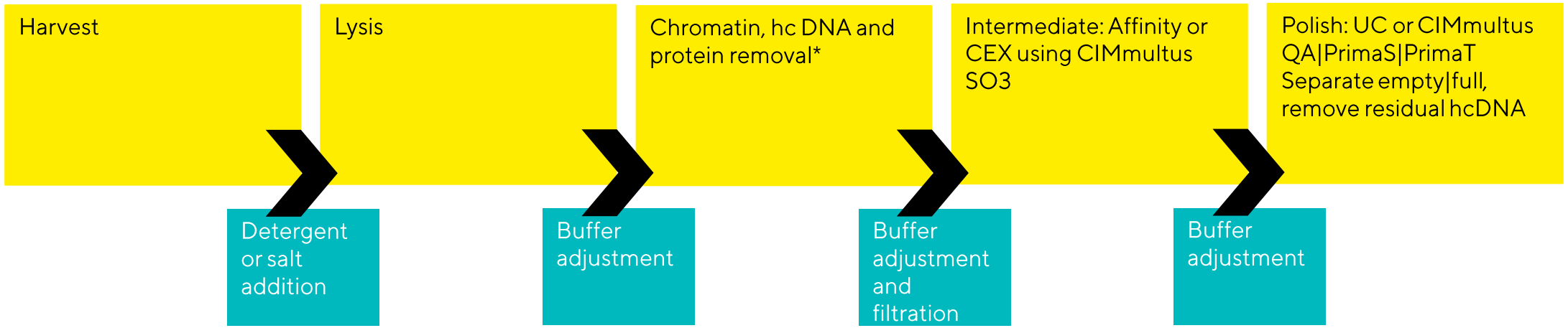
Product impurities are one of the key reasons for treatment side effects. High purity is therefore mandatory for product safety.

Case Study: SBIAS Process Development From CPI to Market Supply in Just 2 Years

2015	2016 to Mid 2018	April 2018	Mid 2018	Current
AveXis finishes Clinical Phase I on Zolgensma. FDA clears AveXis to skip Phase II in order to commercialise and go-to-market quickly	BIA begins to co-develop a fully commercial AAV process development solution for AveXis	AveXis is acquired by Novartis	AveXis starts the production of Zolgensma for the market	Columns supply and further process development

AAV Manufacturing Process, Far From Any Platform at This Point

Usual Steps in AAV DSP Manufacturing Process



*Multiple options to remove Chromatin, alone or combined:

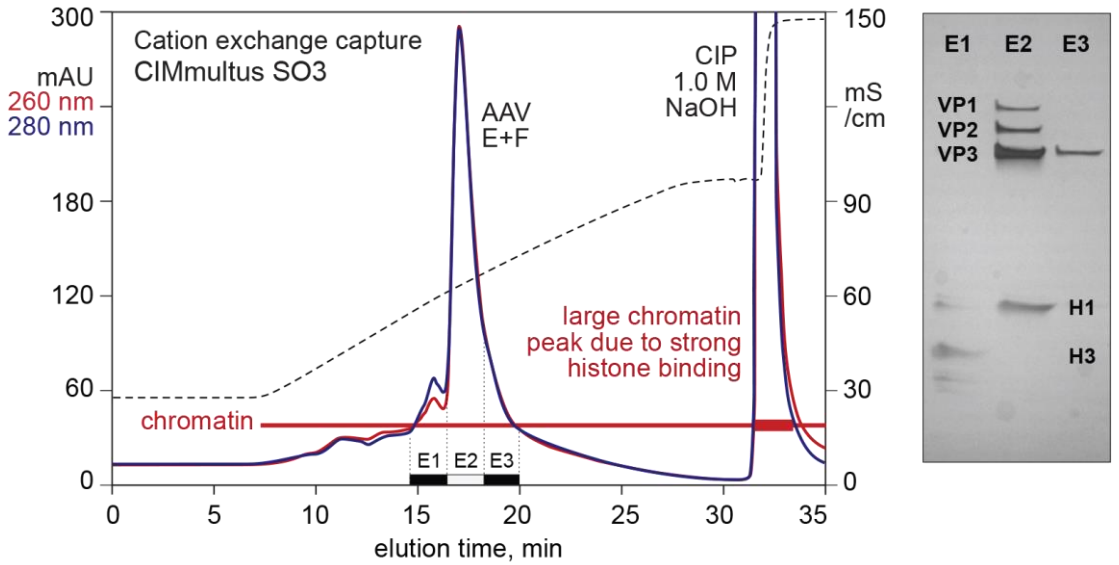
- Flocculation
- TFF/DNAse treatment
- Solid phase extraction

Samples with high AAV titer: Acidic precipitation and direct load on the SO3 column

Capture of AAV by Cation Exchange Column – Works With All Serotypes, Better Than Affinity

Residual Chromatin left after solid phase extraction binds strongly to SO_3 because of its histone component, but it carries a lot of DNA with it.

Removing DNA enables better separation of empty and full capsids.



CIMmultus™ SO3, 1 mL, 2 µm channels, 10 CV/min

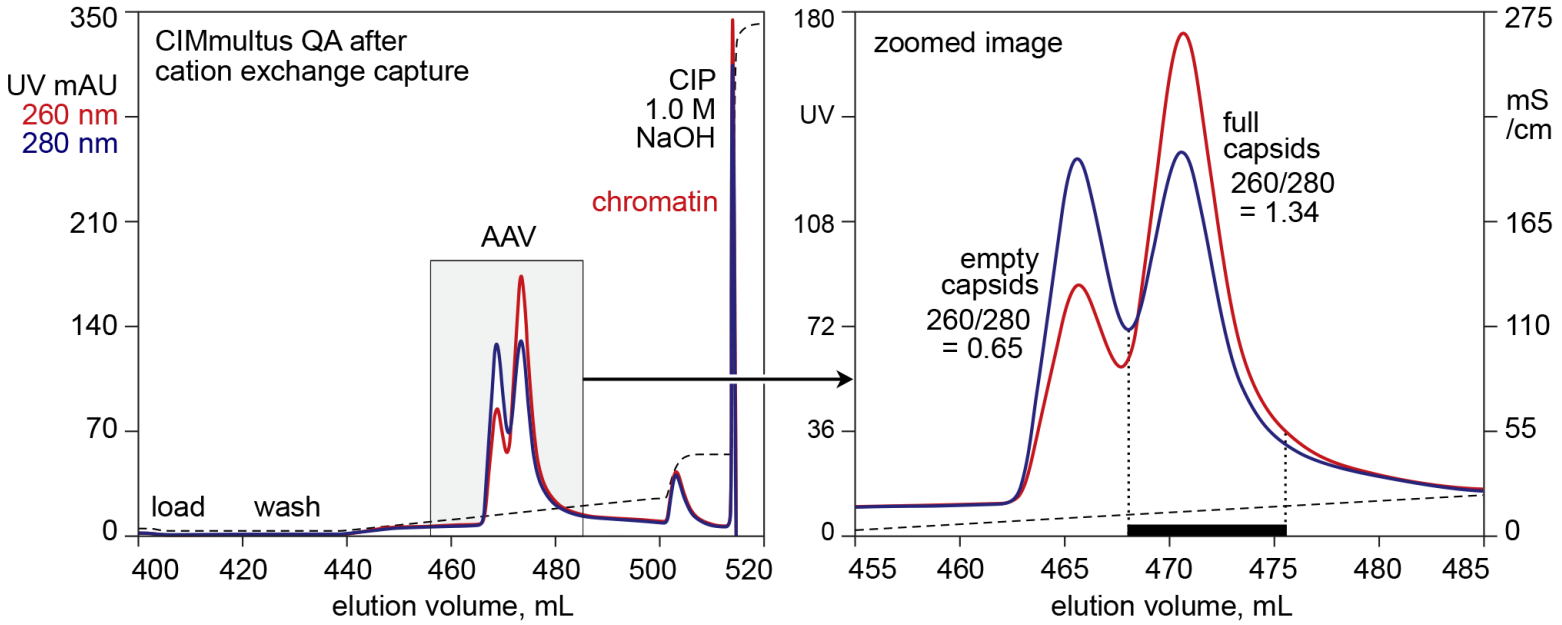
CIMasphere treatment also lowers viscosity and improves filterability, so that low titre samples can be concentrated much more effectively.

Comparing to the affinity capture the SO_3 offers*:
- **10% improved process recovery** analyzed by dPCR and PATfix HPLC, from clarification to the polishing step
- Comparable impurities reduction
- Reduced processing time

*white paper on request

AAV Empty|Full Separation Method Used So Far

CIMmultus SO_3 pre-purified sample loaded on the CIMmultus QA column



CIMmultus QA, 1 mL, 2 µm channels, 10 CV/min (600 CV/h).

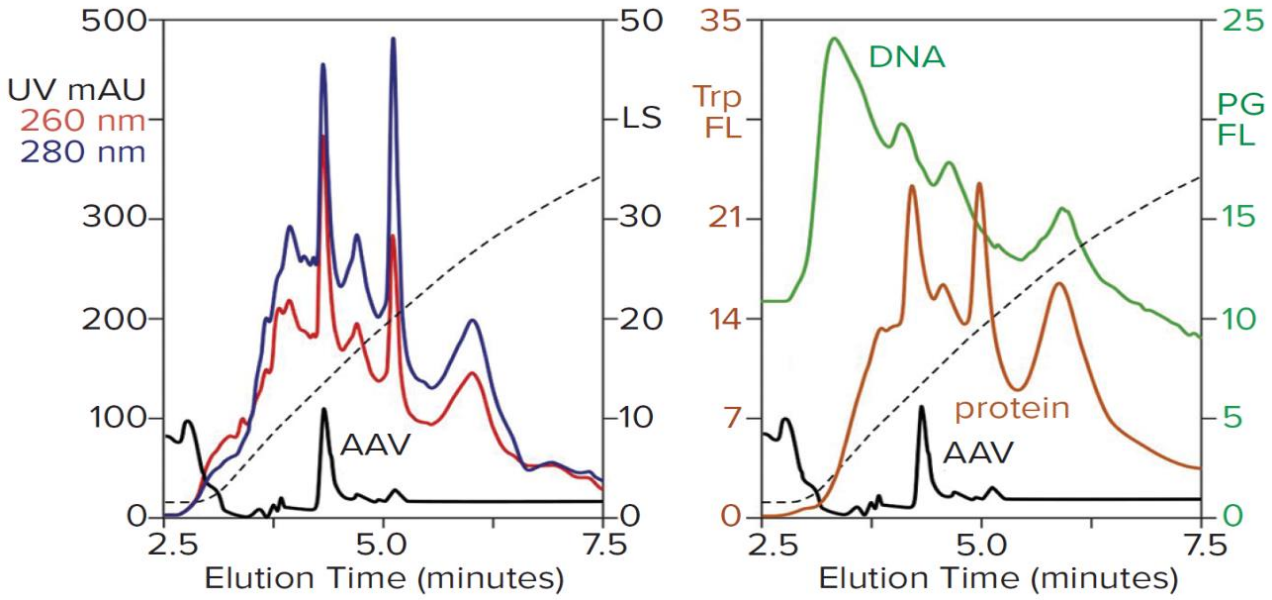
Separation of empty and full AAV capsids by anion exchange chromatography with a salt gradient is described in US patents US9198984B2 and US20160040137A1.

Gagnon et al., 2020. BioProcess International 18(11-12)S.

PATfix System Enables Fast Process Development and Orthogonal Process Control

PATfix System For Faster Process Development and In-Process Control

PATfix LC system with multiple detectors allows for sample characterisation in an hour.



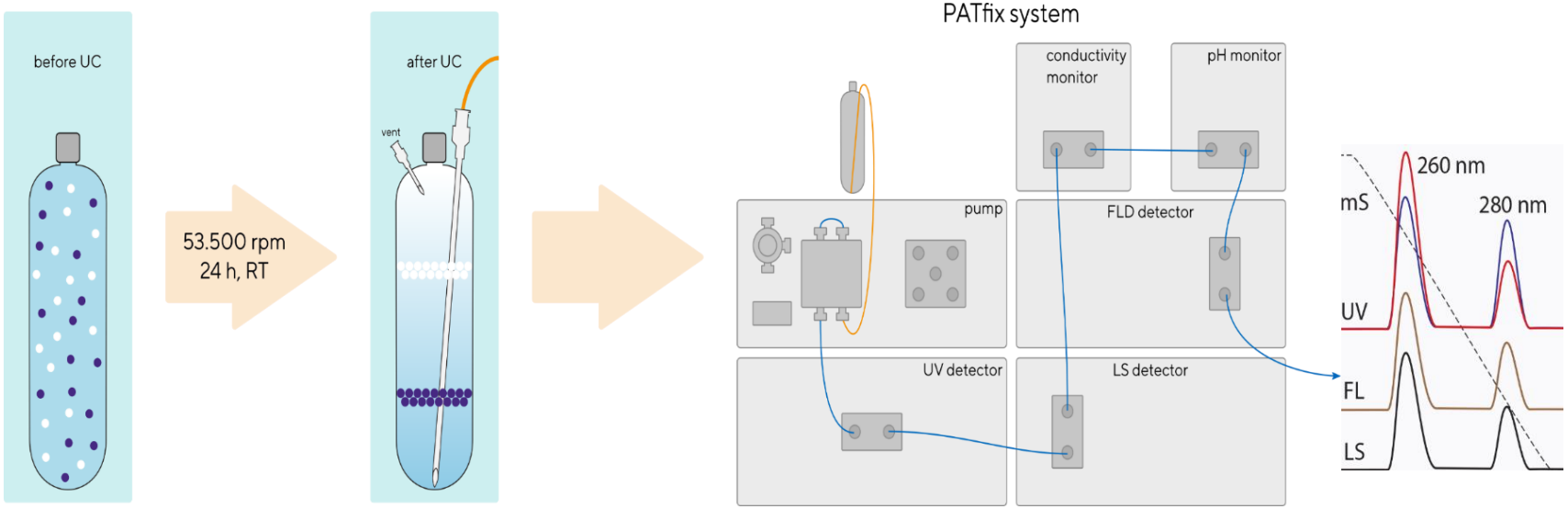
Optional: novel, very reliable and affordable **MALS detector**

Cation exchange does not discriminate empty from full capsids but it still provides fast characterization of total AAV and contaminant content. UV wavelength ratios provide a hint about relative DNA and protein distribution but **fluorescence enables direct quantitative comparison**.

Ultracentrifuge Fractions Collection and Analysis Using PATfix Detectors

Density gradient fractionation followed by stratigraphic analysis through the PATfix detector array, orthogonal to liquid chromatography

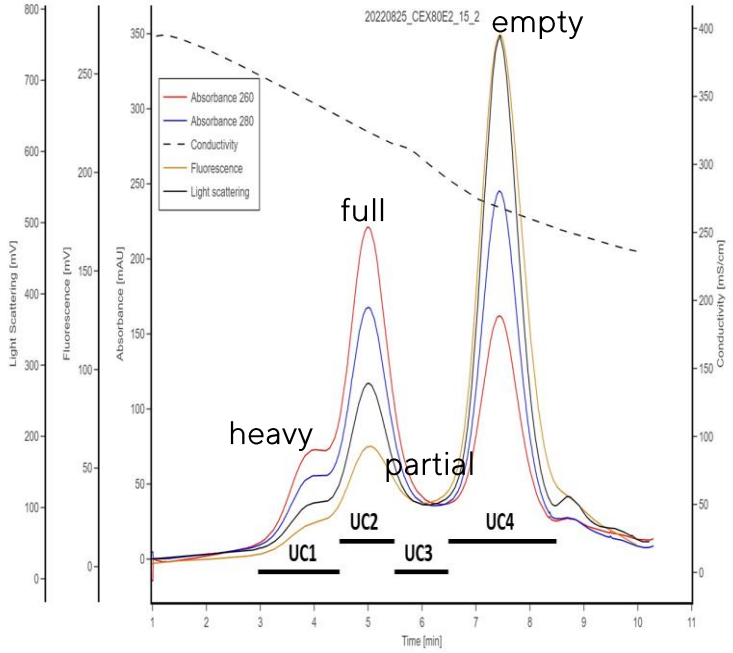
Should not be mixed with AUC method



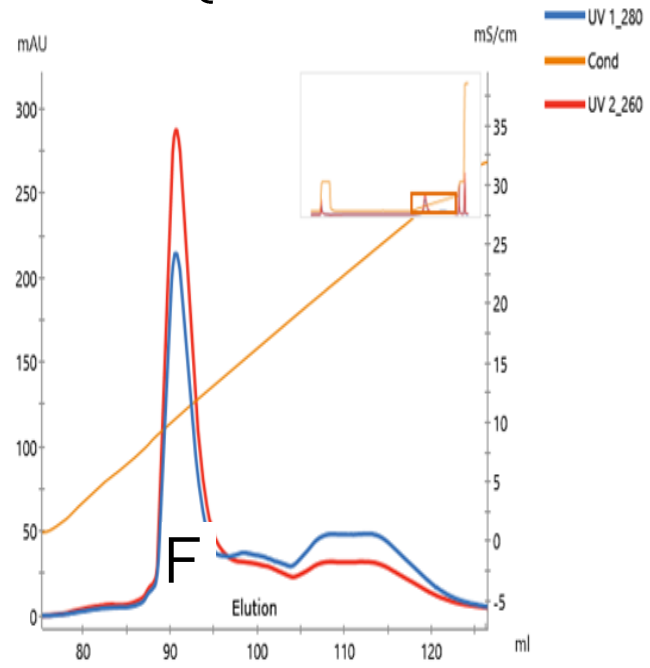
Peljhan et al, 2021. Cell & Gene Therapy Insights. DOI: 10.18609/cgti.2021.039.

Cimmultus SO_3 Purified AAV9 Sample, Further Purified Using UC or AEX

UC fractionation

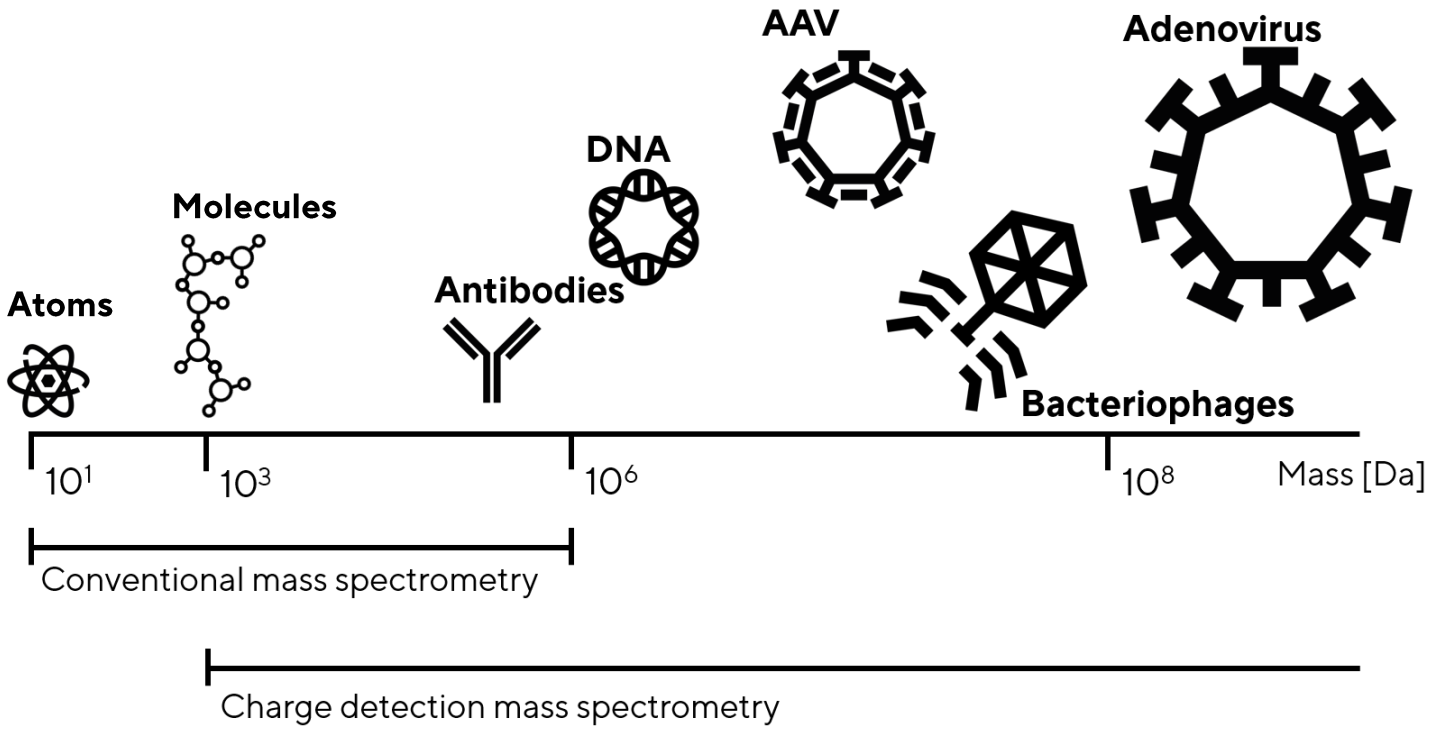


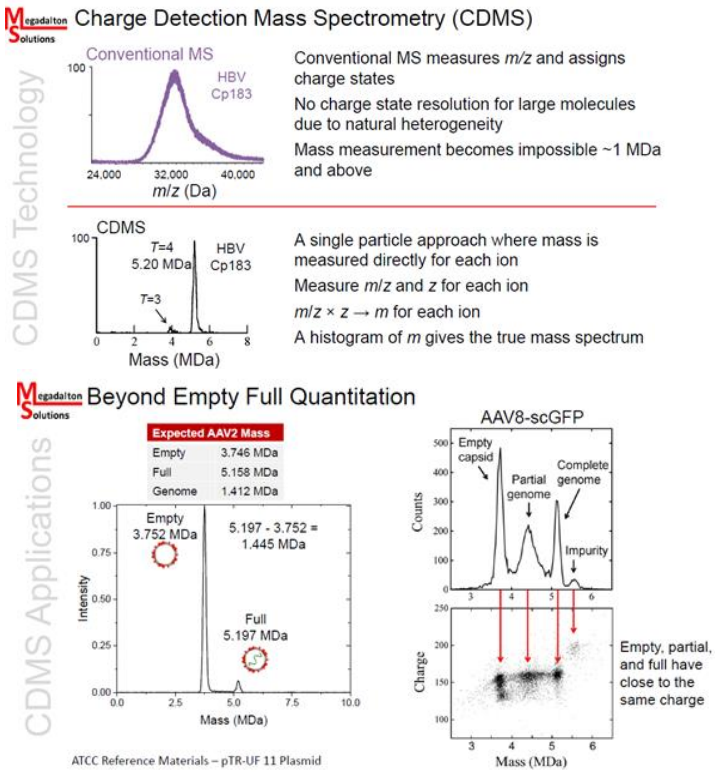
CIMmultus QA elution



All samples were concentrated and buffer exchanged to 50 mM TRIS, 150 mM NaCl, 2 mM MgCl2, 0.1% Poloxamer 188, pH 7.5

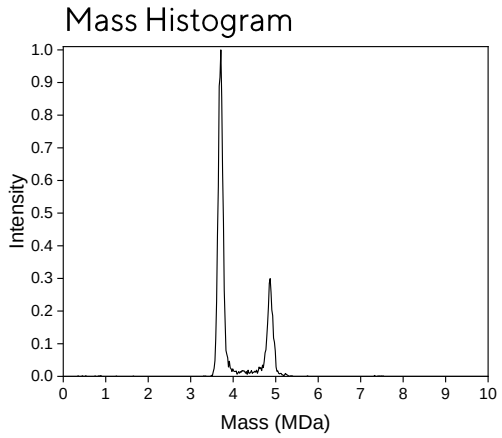
Analysis of AAV Capsids Using Charge Detection Mass Spectrometry (CDMS)

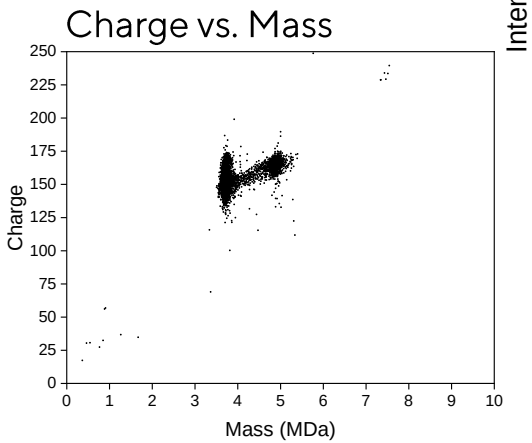


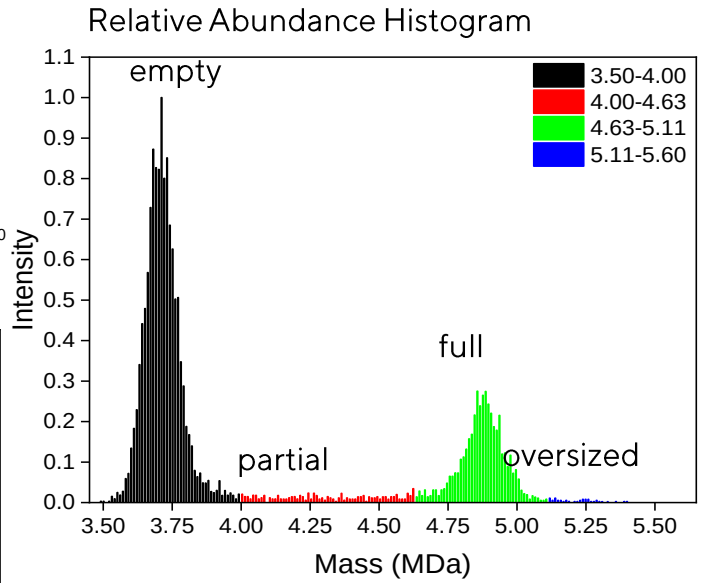


CDMS of SO_3 Eluate, Empty More Heterogenous Than Full

Total Ions Measured:	5999		
Relative Abundances		Average Charge	Average Mass
<3.50 MDa (%)	0.2	46.1	1.31
3.50-4.00 MDa (%)	70.1	152.0	3.73
4.00-4.63 MDa (%)	4.4	156.8	4.31
4.63-5.11 MDa (%)	24.6	164.9	4.88
5.11-5.60 MDa (%)	0.7	163.7	5.22
>5.60 MDa (%)	0.1	234.6	7.20
Peak Fitting:			
Empty (MDa)	3.70	σ (MDa)	0.06
Partial (MDa)	N/A	σ (MDa)	N/A
Full (MDa)	4.87	σ (MDa)	0.07

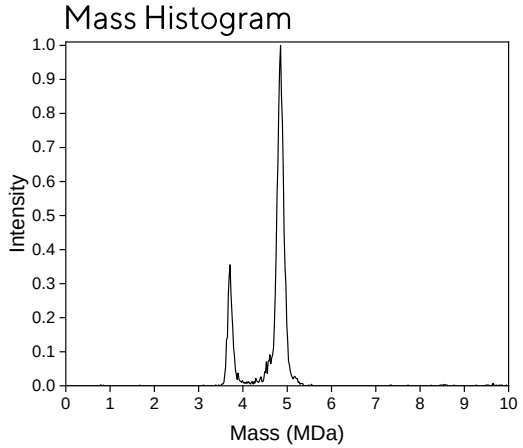


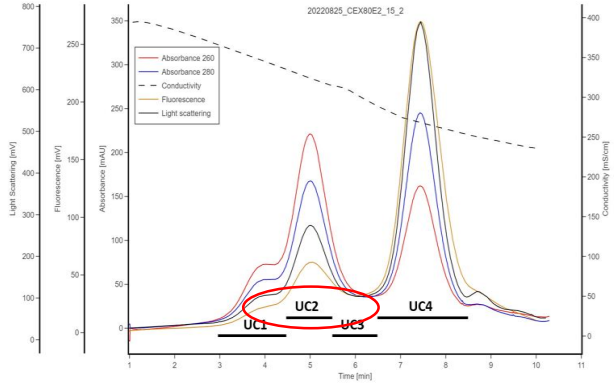


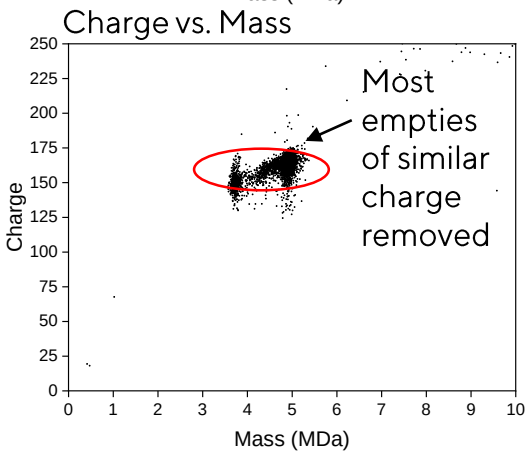


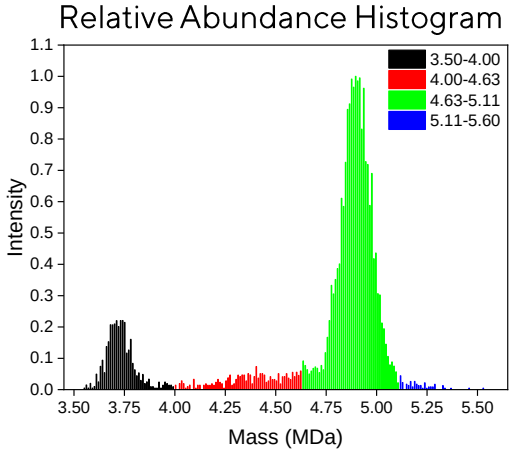
CDMS of UC 02 (“Full”) Consists of Many Other AAV Capsids

Total Ions Measured:	5363		
Relative Abundances		Average Charge	Average Mass
<3.50 MDa (%)	0.1	35.0	0.64
3.50-4.00 MDa (%)	13.5	150.0	3.75
4.00-4.63 MDa (%)	7.5	157.3	4.39
4.63-5.11 MDa (%)	76.8	164.4	4.90
5.11-5.60 MDa (%)	1.4	167.1	5.21
>5.60 MDa (%)	0.7	259.0	9.19
Peak Fitting:			
Empty (MDa)	3.71	σ (MDa)	0.06
Partial (MDa)	N/A	σ (MDa)	N/A
Full (MDa)	4.89	σ (MDa)	0.07



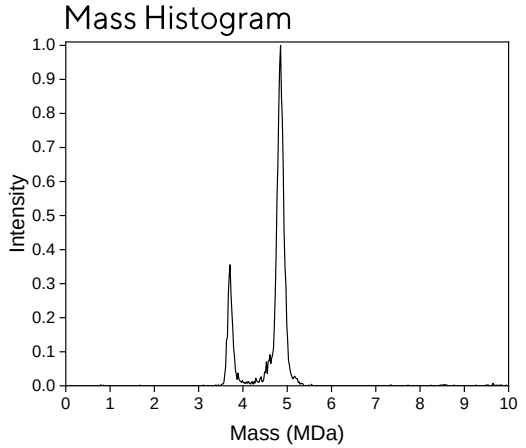


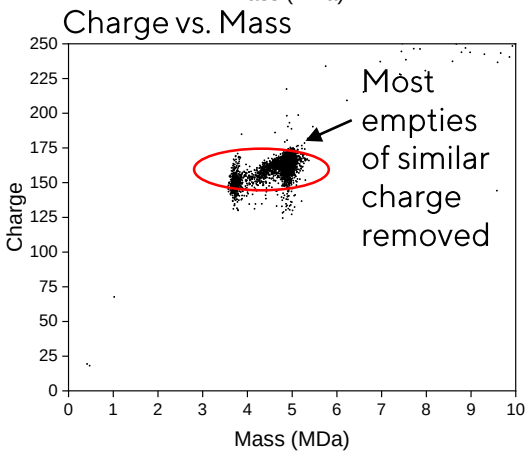


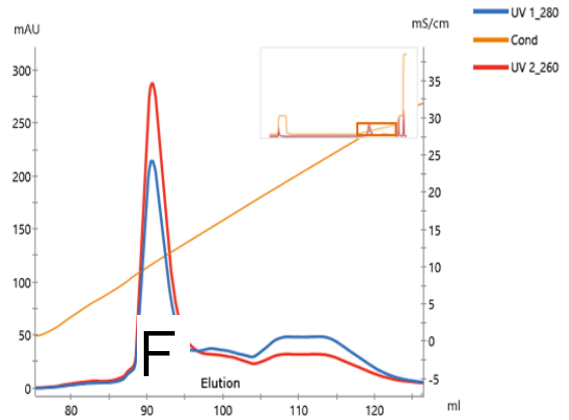


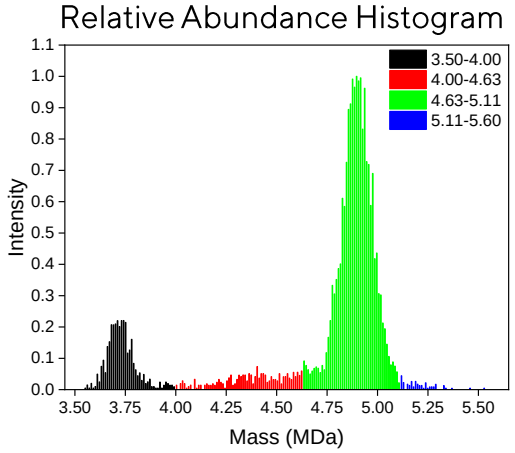
CDMS of the Cimmultus QA Full Fraction Consists of Many AAV Capsids

Total Ions Measured:	6790		
Relative Abundances		Average Charge	Average Mass
<3.50 MDa (%)	0.0	56.3	1.06
3.50-4.00 MDa (%)	12.3	150.6	3.77
4.00-4.63 MDa (%)	8.1	155.9	4.32
4.63-5.11 MDa (%)	73.9	163.0	4.96
5.11-5.60 MDa (%)	5.3	165.4	5.18
>5.60 MDa (%)	0.3	269.1	8.90
Peak Fitting:			
Empty (MDa)	3.73	σ (MDa)	0.07
Partial (MDa)	N/A	σ (MDa)	N/A
Full (MDa)	4.96	σ (MDa)	0.08





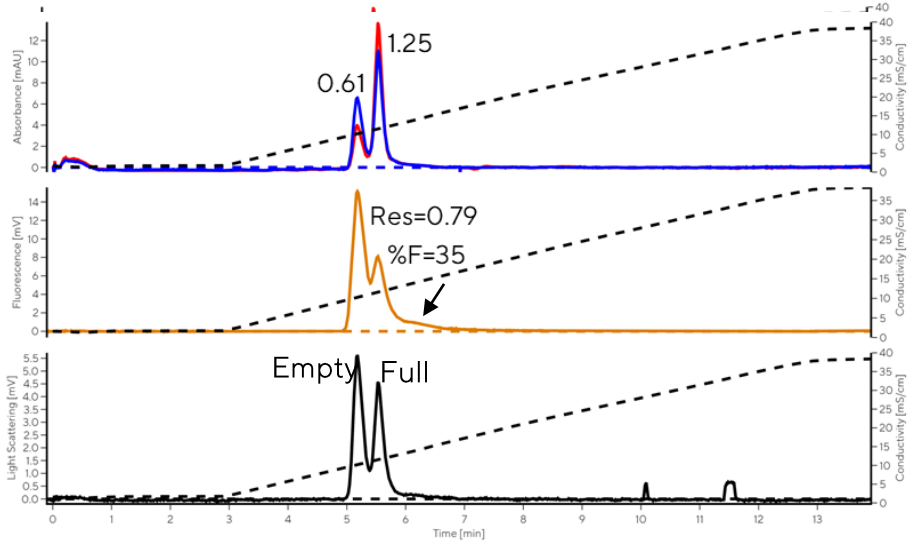




New Empty, Partial, Full and Heavy AAV Capsids Separation Methods Developed

Novel High Resolution Method to Separate Different AAV Capsids Now Available

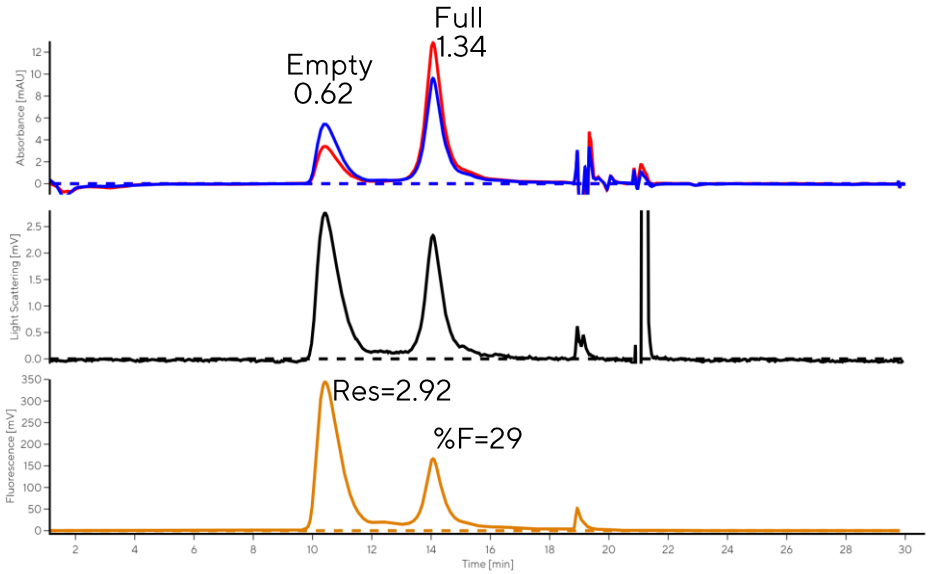
Standard QA method
(0-400 mM linear NaCl 100 CVs gradient; pH 9.0)



Detectors:
- UV 260 nm
- UV 280 nm
- FLD Trp
- MALS 90°

Method optimization

Novel QA method*



Standard Method:

- Low resolution between full AAV and other inactive capsids
- 3 species observed
- Need metod for better characterization of AAV sample heterogeneity

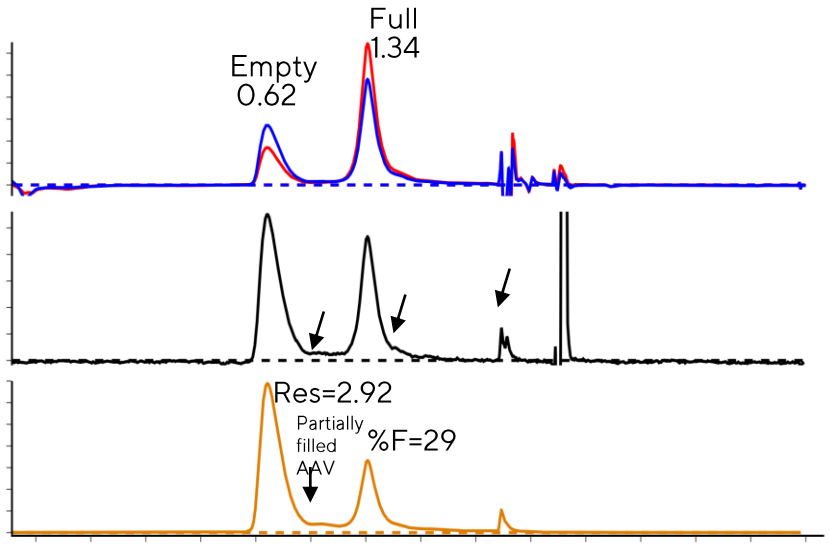
Novel Method:

- Significantly enhanced Empty/Full resolution
- At least 5 species observed
- Fractionation of AAV capsids

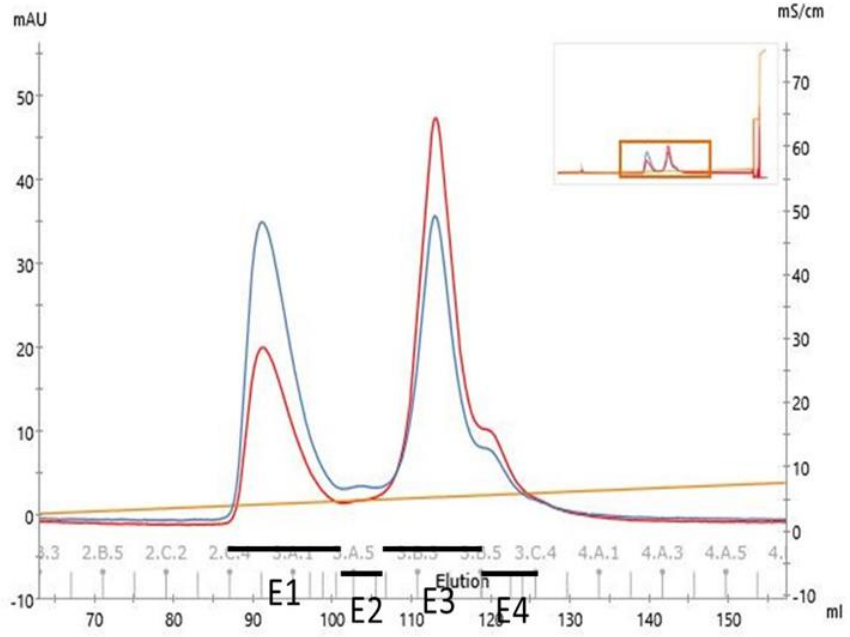
***Patent pending**.

Scale-up of the Novel High Resolution Method to Separate Different AAV Capsids

Novel QA method*



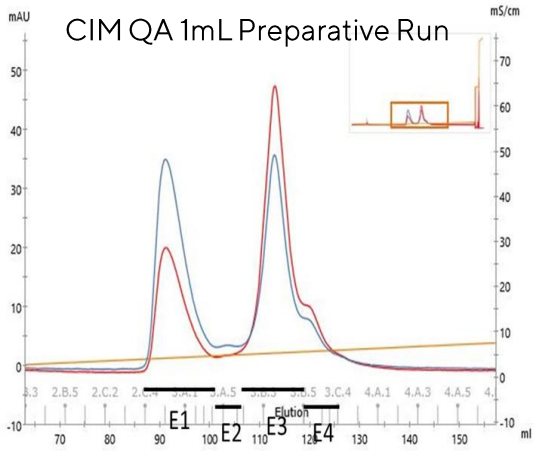
Scale-up



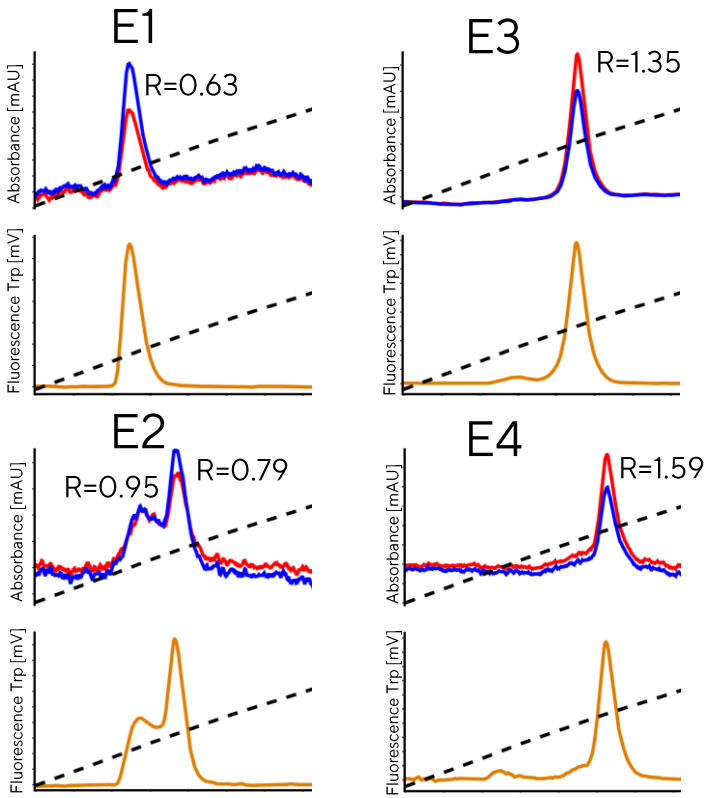
Fractions collected and analysed using PATfix

*Patent pending.

Analysis of the Scale-up Fractions Using Orthogonal Methods



PATfix fraction analysis



Detectors:
Red – UV 260 nm
Blue – UV 280 nm
Orange – Trp FLD
Black – MALS

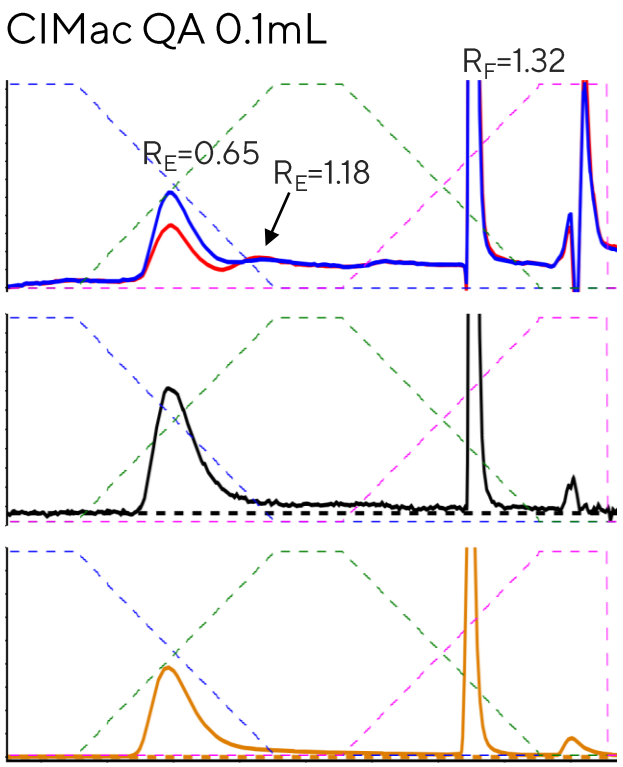
dPCR Results

Sample	Volume (mL)	Average concentration (vg/ml)	Final concentration (vg/ml)	Recovery (%)
5f_QA_23_02 LOAD	258,45	1,87E+10	4,82E+12	100%
5f_QA_23_02 FT	258,90	Below LOQ	Below LOQ	Below LOQ
5f_QA_23_02 W	10,00	Below LOQ	Below LOQ	Below LOQ
5f_QA_23_02 E1	16,10	4,90E+08	7,89E+09	0%
5f_QA_23_02 E2	6,85	1,95E+10	1,33E+11	3%
5f_QA_23_02 E3	11,80	2,83E+11	3,34E+12	69%
5f_QA_23_02 E4	12,00	3,90E+10	4,68E+11	10%

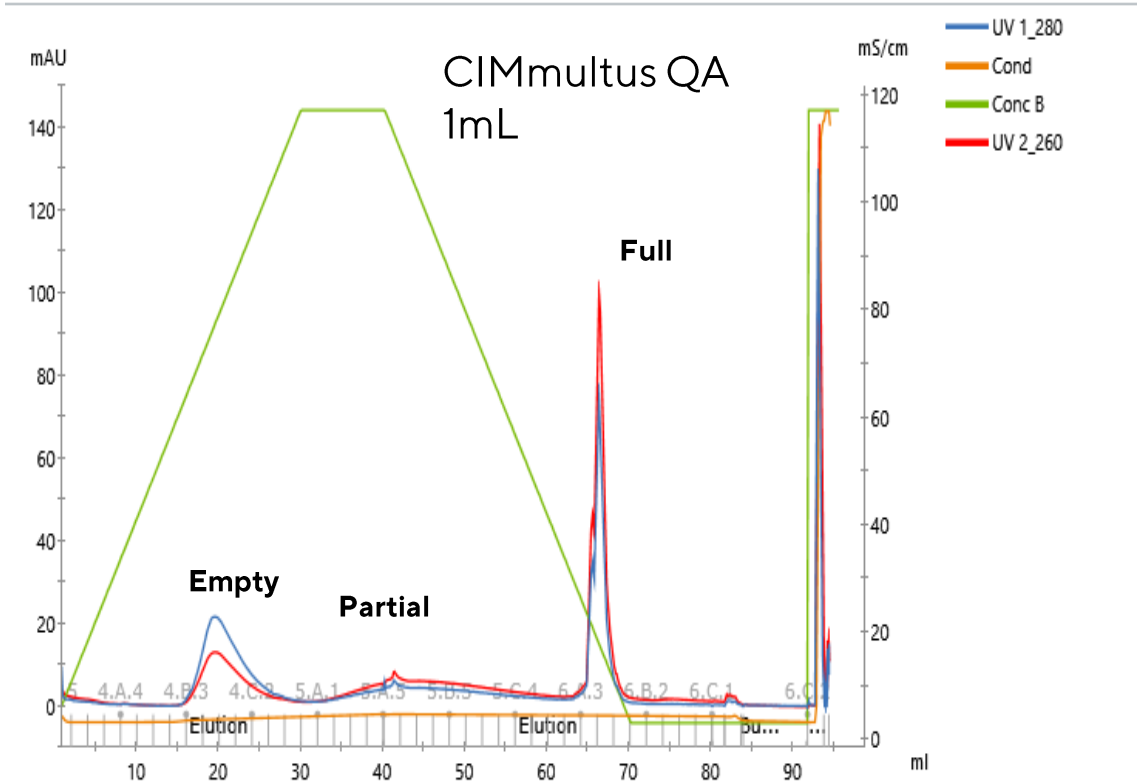
By UV >95%, by FLD >90% AAV full capsid

Further Improvement of the Novel High Resolution Method to Separate AAV Capsids*

Detectors:
Red – UV 260 nm
Blue – UV 280 nm
Orange – Trp FLD
Black – MALS

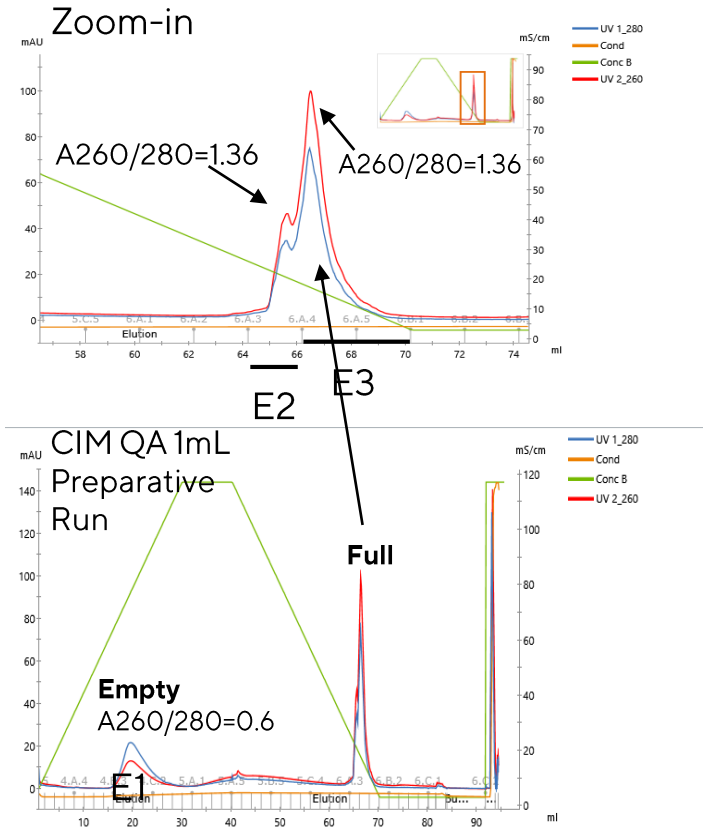


Scale-up

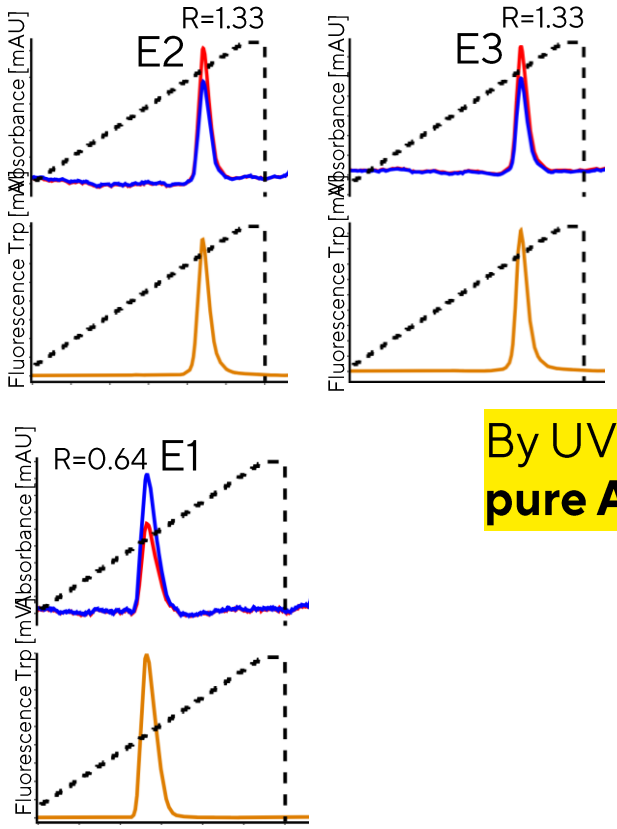


***Patent pending**.

Analysis of the Scale-up Fractions Using Orthogonal Methods



PATfix fraction analysis

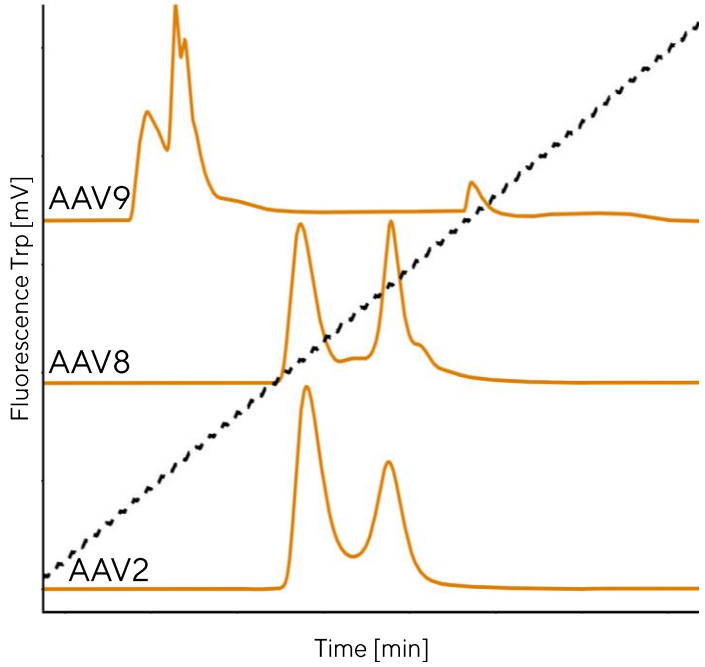


Detectors:
- UV 260 nm
- UV 280 nm
- Trp FLD
- MALS

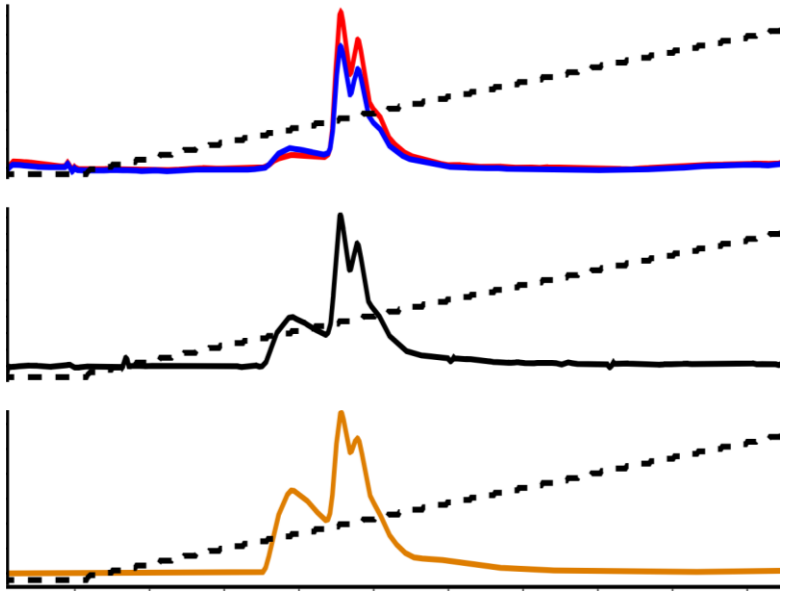
By UV and FLD **> 99% pure AAV full capsid**

Novel Method Works with AAV8 and AAV2 Capsids, the AAV9 Needs Fine Tuning

CIM SO_3 purified samples of AAV serotypes 2, 8 and 9



Further optimization improves the separation of the AAV9 capsids



Not shown yet, much **better resolution** is achieved by adding **additional species** in the **buffers.**
Will be presented after the next patent application is filed.

Business Model for Using New Methods

First option:

You wait for the patents applications **publishing 4th Q/2024, 1st Q/2025** and develop the method after by yourself.

Second option:

- **Feasibility study is performed free of charge** to demonstrate that the methods provide the expected high-resolution separation using client's AAV sample. Outcome of this study is a proof of principle and consists only of chromatograms and a basic interpretation of the characteristics of the sample based on the chromatograms. Fractions can be sent to the client for further evaluation.

- Based on the feasibility study data Sartorius BIA Separations prepares an offer to develop the method optimised to enrich the client's full AAV capsid. If the offer is accepted and MSA service contract is signed Sartorius BIA Separations develop the method. After payment for the service work **client is granted a worldwide non-exclusive, free of charge, license for using novel CIM QA HR empty|full methods.**

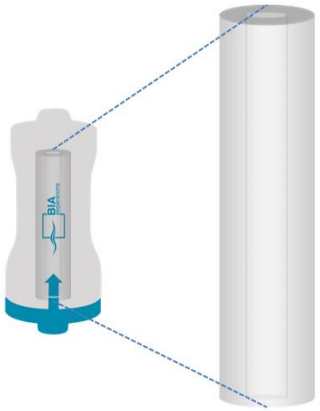
New CIMmultus Columns HR (High Reproducibility) Column Release Criteria, With the 1st Batch of AAV8: [KCl] Empty = 92.3 mM ± 3%

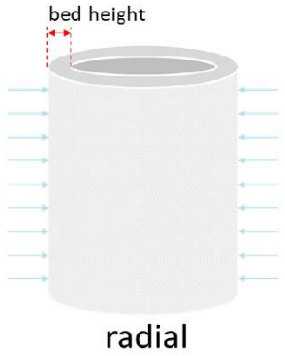
Cim Monoliths Are Single Piece of Highly Channelled Polymer Packed In CIM Housing

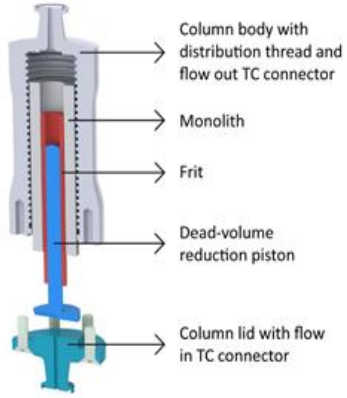
Monoliths are a single-unit structures with highly interconnected convective channels distributed homogeneously throughout the entire bed.

Polymethacrylate monoliths with different channel size 1.3, 2 and 6 µm in diameter and void volume of 60-65%.

Radial geometry enables higher volumetric throughput by increasing the intake surface area, and decreasing the bed height („thickness of tube“).







Laminar flow
= no shear forces

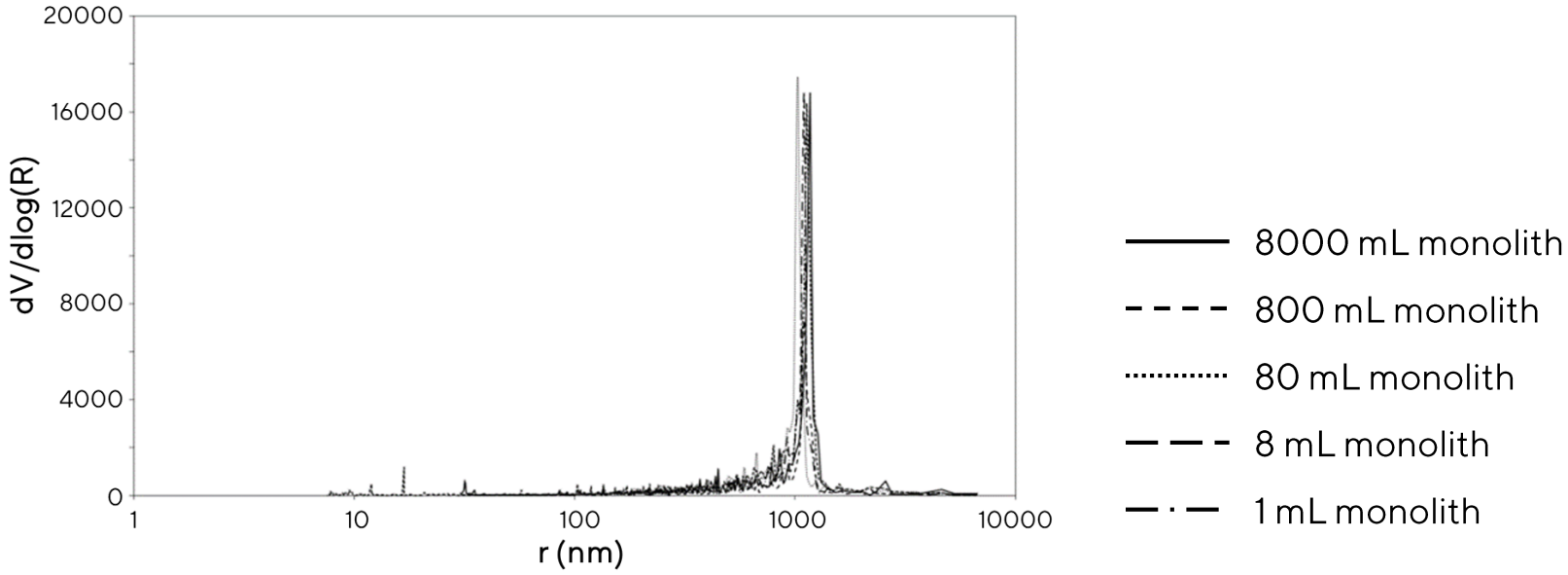
No dead end pores
= no diffusion

Low backpressure
= high flow rate

Large channels
= no entrapment

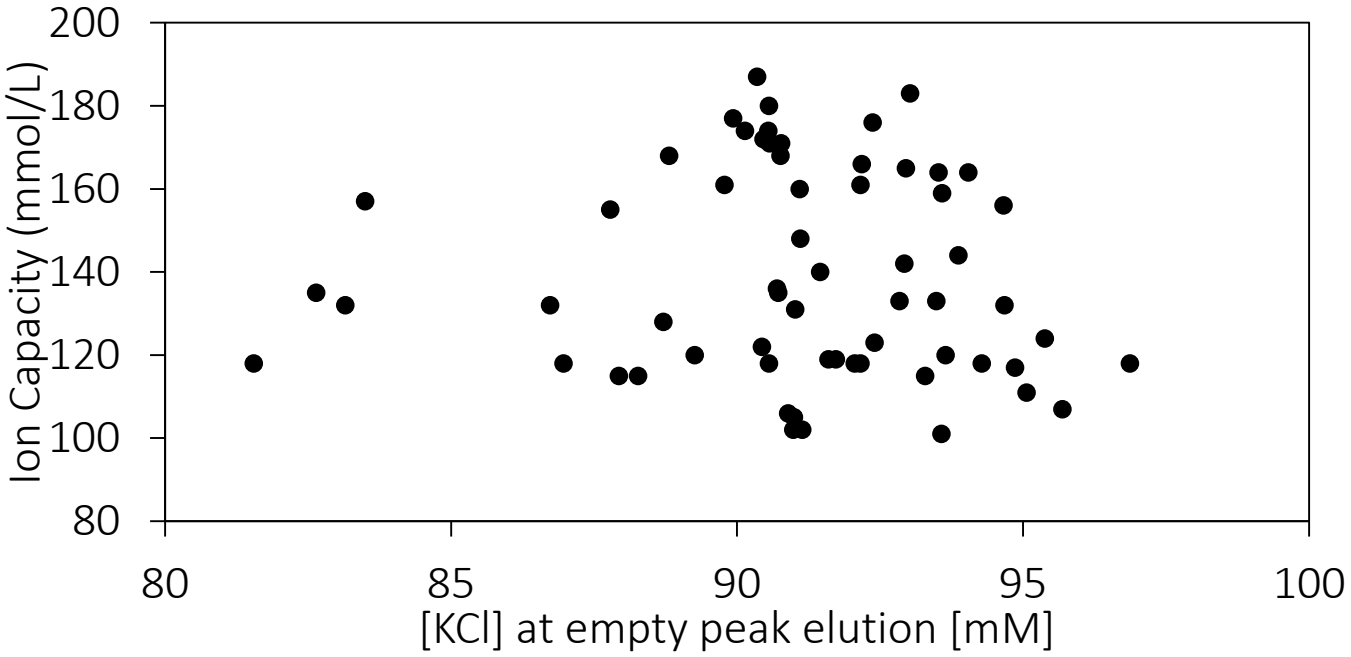
Pore Size Distribution and Accessible Inner Surface Areas

The monoliths have a **monomodal pore size distribution demonstrated** through **Hg porosimetry measurements** (our standard QC method). An example of pore distribution for monoliths of different sizes is shown in the figure below.

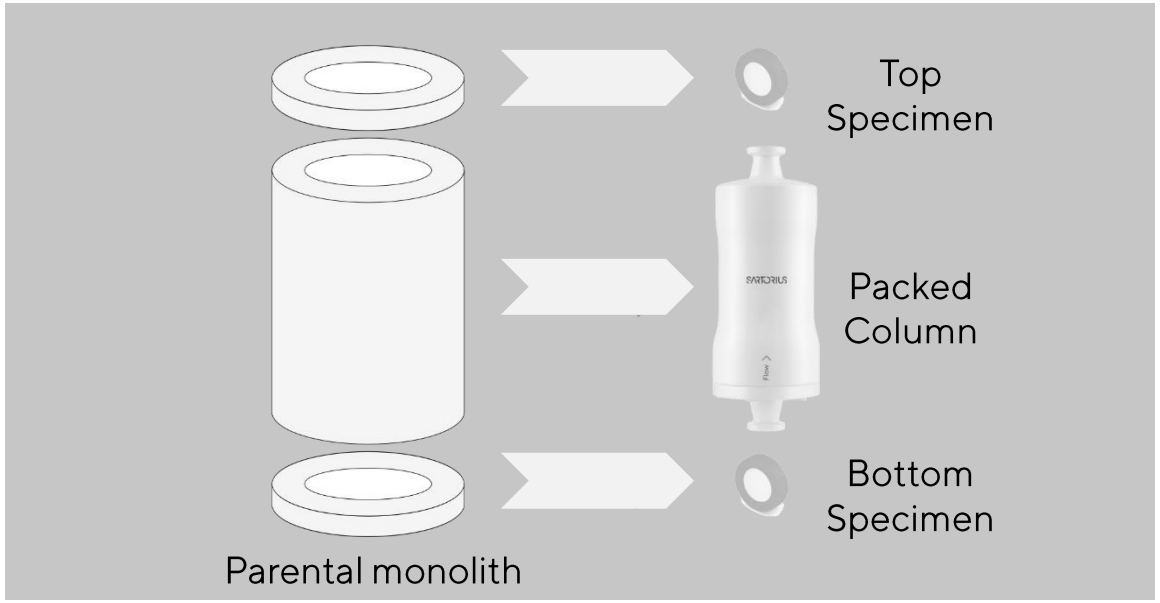


Comparison of the channel size distribution for the samples of Epoxy monoliths with average channel radius 950 nm – 1150 nm taken from different monolith sizes

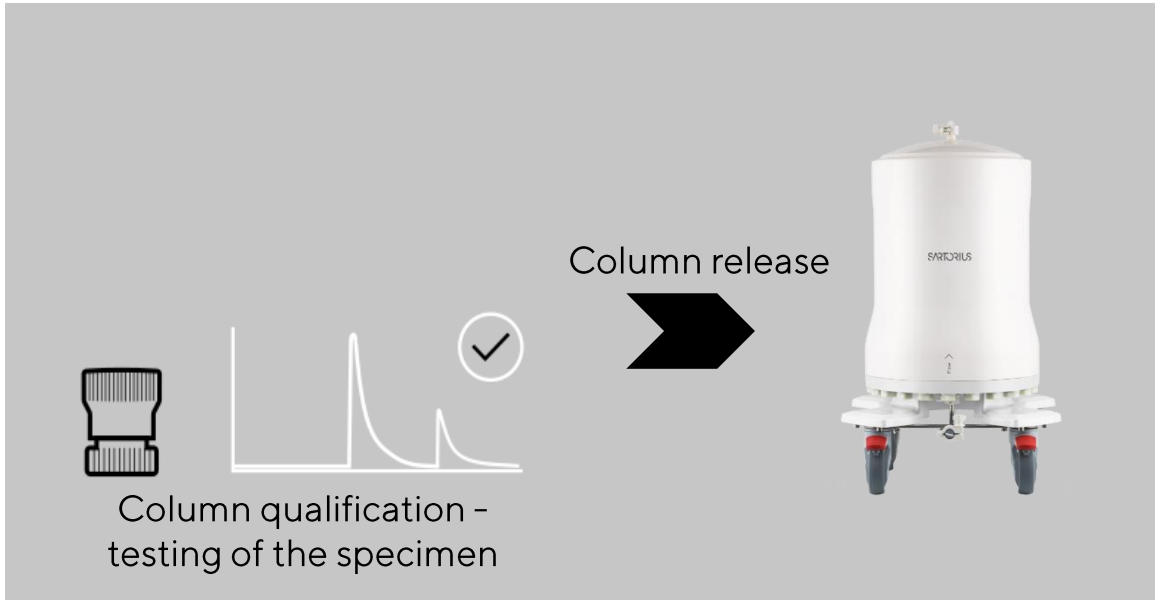
No Correlation Between Ionic Capacity and AAV Empty Capsid Elution



Specimen – Representative Part of the Cimmultus Columns Larger Than 40ML



- 200 µL unit derived from parental column
- Available for 40 ml columns and larger
- Customer receives 1 specimen columns, additional units available on request

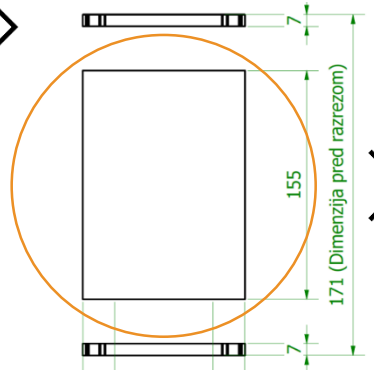


- Enables column release according to the set acceptance criteria (QC testing of monolithic column with AAV before release)
- Allows for qualification of the column by customer prior use
- AAV method and AAV standard for qualification available

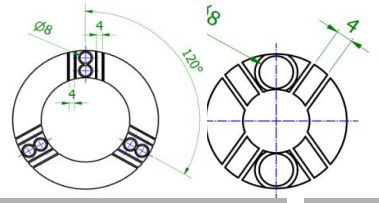
Specimen | Representative Part of the CIM Column

Polymerization and modification of 80, 800, 8000 mL monoliths

Cutting monolithic rings above and below the final column dimensions

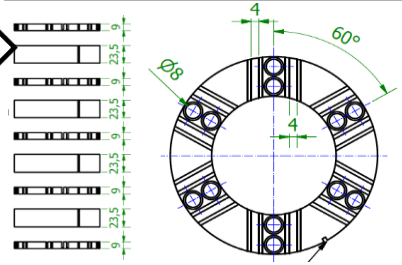


Preparing 0.2 mL disks out of the rings = **control disks** (Specimen)



Packing and chromatographic testing of 80, 800, 8000 mL columns

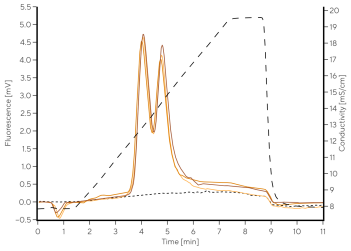
Unpacking and cutting the rings along the column height, followed by 0.2 mL disk drilling from different positions



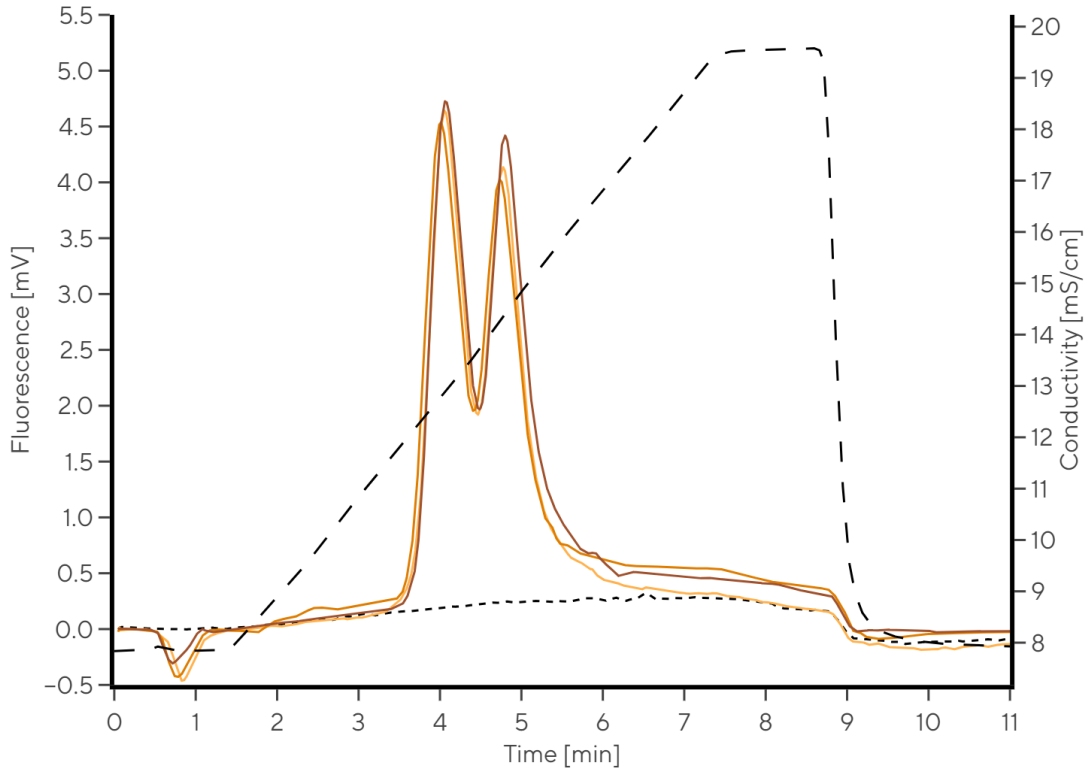
Testing **control disks** (Specimen):
- Chromatography
- Mechanical properties

Testing **drilled disks**:
- Chromatography
- Mechanical properties

Data evaluation



Scale-Up: Iso-Conductivity Approach (Gradient Duration in CV Is Preserved)



baseline
Specimen from 80 mL monolith
Specimen from 800 mL monolith
Specimen from 8000 mL monolith
gradient

Test columns: 0,2 mL Specimen CIM QA HR

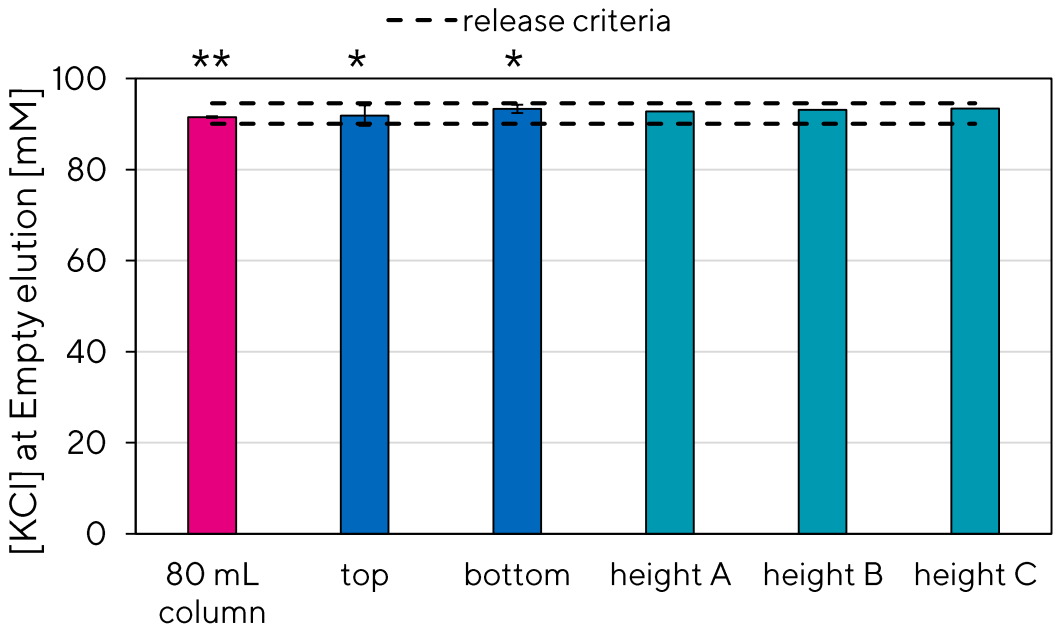
System: PATfix and column thermostat at 23.0±0.5°C

Sample: internal AAV8 standard

Method: linear KCl gradient over 30 CV at 1 mL/mL

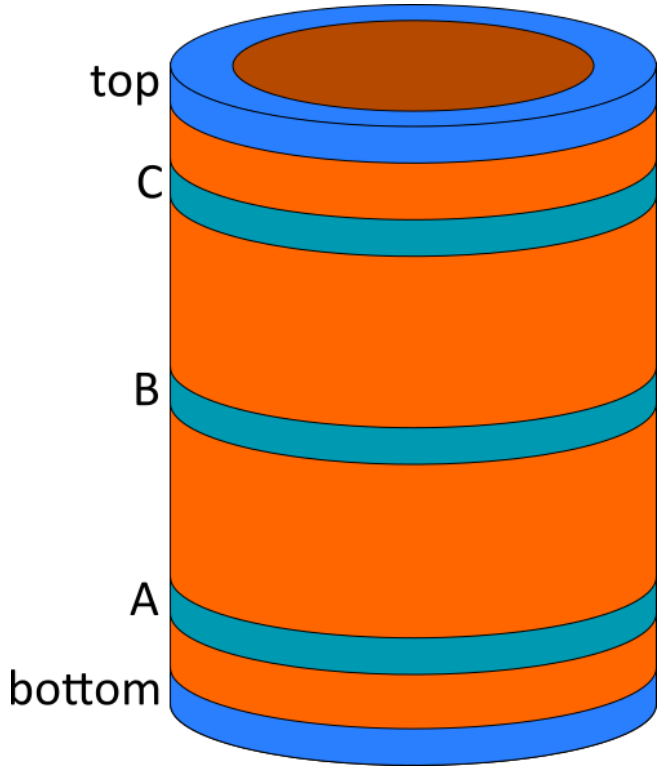
Detection: Fluorescence and conductivity detectors

CIMmultus QA 80 mL Column Homogeneity for Empty|Full AAV Separation

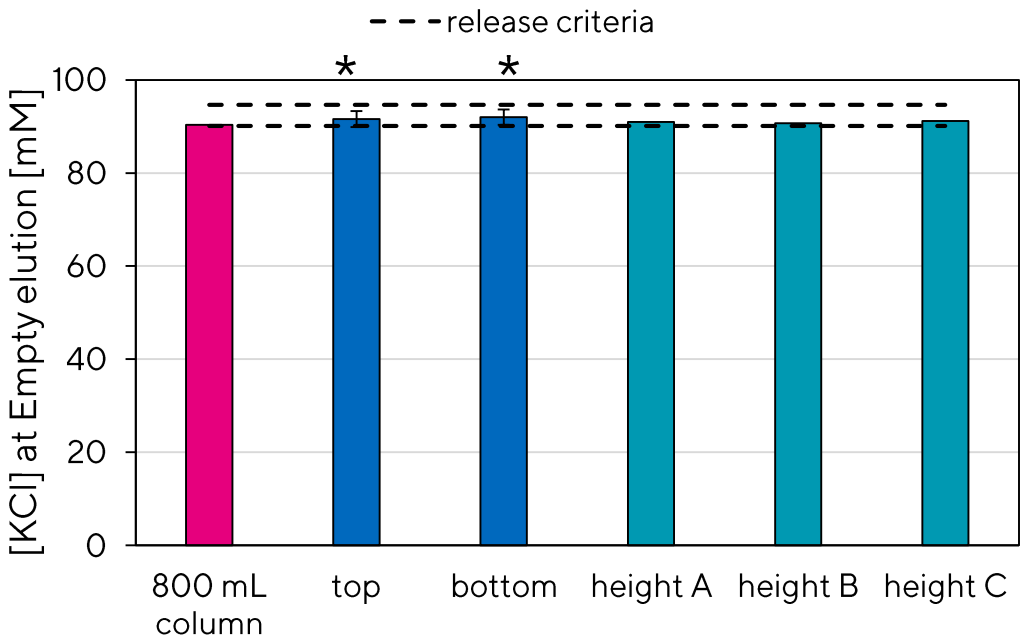


$[KCl]_{Empty}$ = 92,54 mM ± 1.3% RSD (N = 9)

* N = 2 (2 evaluated specimens from this position)
** N = 2 (2 runs performed on the column)

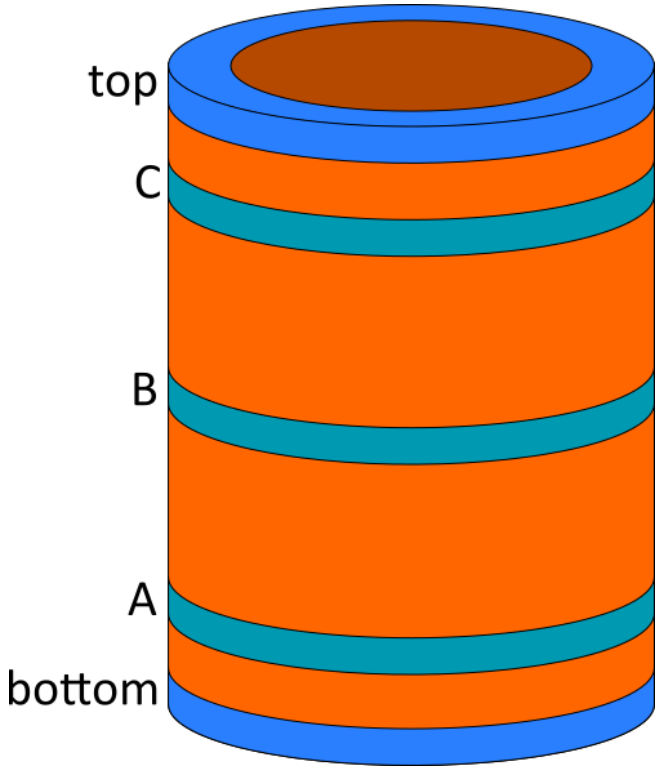


CIMmultus QA 800 mL Column Homogeneity for Empty|Full AAV Separation



$[KCl]_{Empty}$ = 91,29 mM ± 1.2% RSD (N = 8)

* N = 2 (2 evaluated specimens from this position)



HR Line Adjustments: Housing and Monolith Dimensions From 1 to 8 mL Scales

CIMmultus 1 mL

CIMmultus HR 1 mL

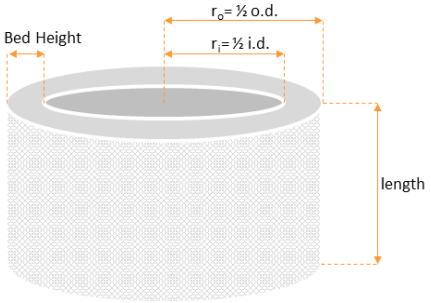
CIMmultus 4 mL

CIMmultus HR 4 mL

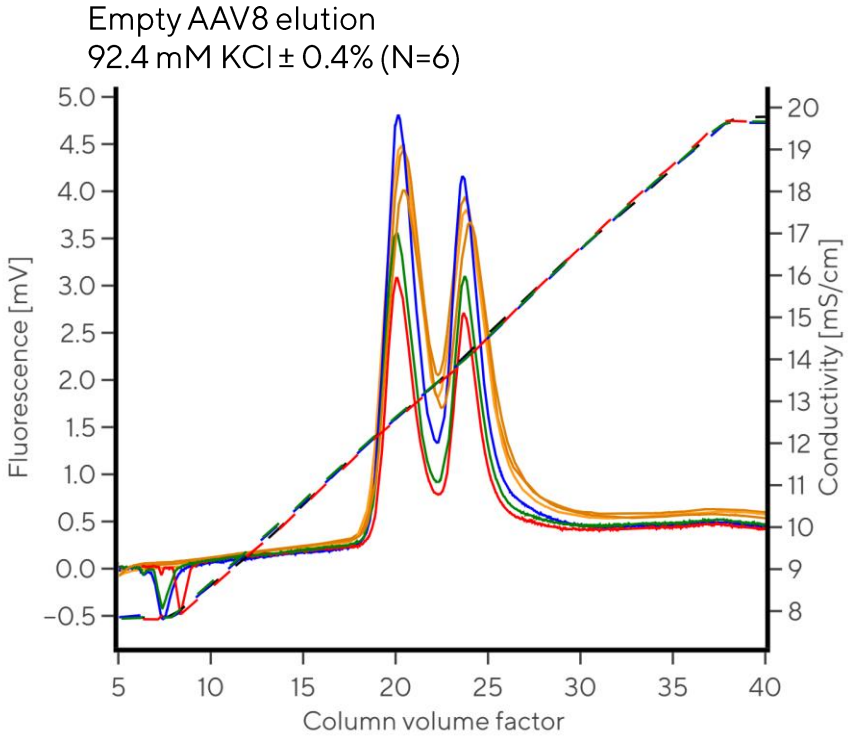
CIMmultus 8 mL

CIMmultus HR 8 mL

Column size	Thickness (i.e. Bed height)	Height
1 mL	0.60 cm	4.2 mm
1 mL HR	**0.60 cm**	**3.8 mm**
4 mL	0.42 cm	28 mm
4 mL HR	**0.60 cm**	**16.3 mm**
8 mL	0.42 cm	56 mm
8 mL HR	**0.60 cm**	**32.6 mm**



Scale-Up: Iso-Conductivity Approach (Gradient Duration in CV Is Preserved)



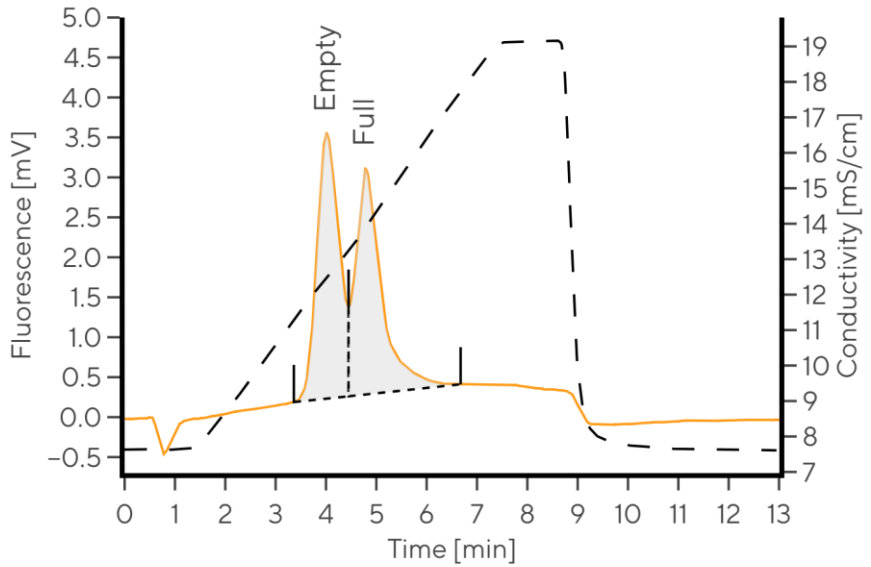
Isoconductivity overlay

- 1 mL
- 4 mL
- 8 mL
- 80 mL Specimen
- 800 mL Specimen
- 8000 mL Specimen
- Conductivity

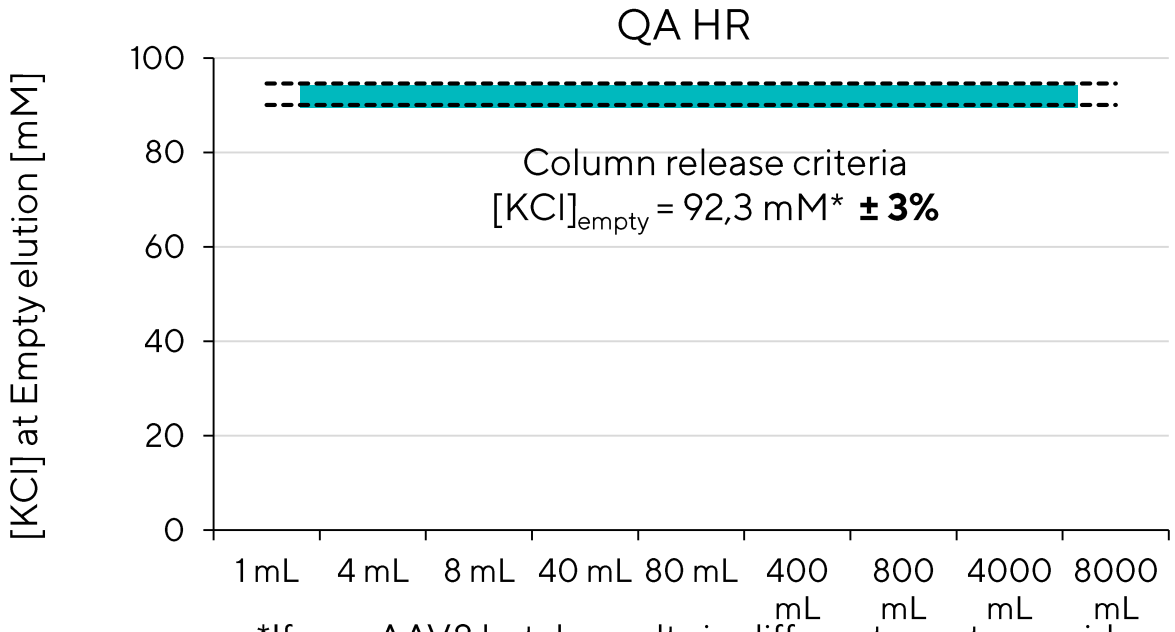
AAV2/8 empty-full separation overlay of:

- 1mL, 4 mL and 8 mL **columns**
- **Specimen** of the 80 mL, 800 mL and 8000 mL columns.

QA HR Release Criteria Based on the AAV8 Empty Capsid Elution



A representative chromatogram of analytical empty/full AAV8 capsid separation on 0.2 mL QA HR Specimen.

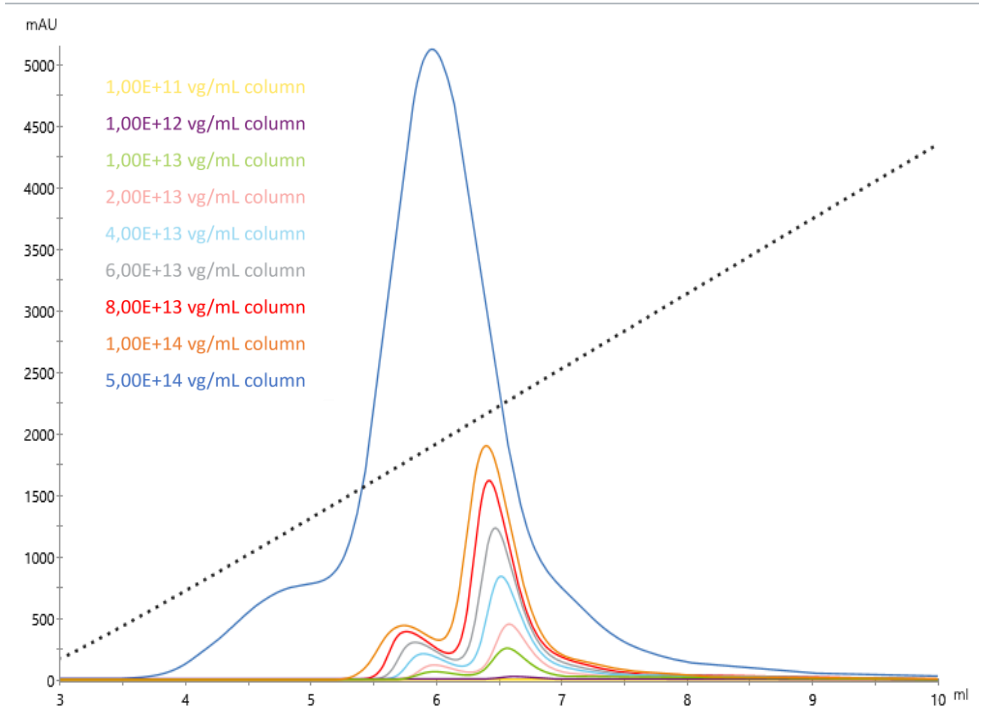


*If new AAV8 batch results in different empty capsid elution a bridge study will be performed.

Each CIM QA HR column is released based on AAV8 empty/full separation.
Column release criteria corresponds to the concentration of the KCl which elutes the Empty AAV2/8 capsid within **the ± 3% range.**

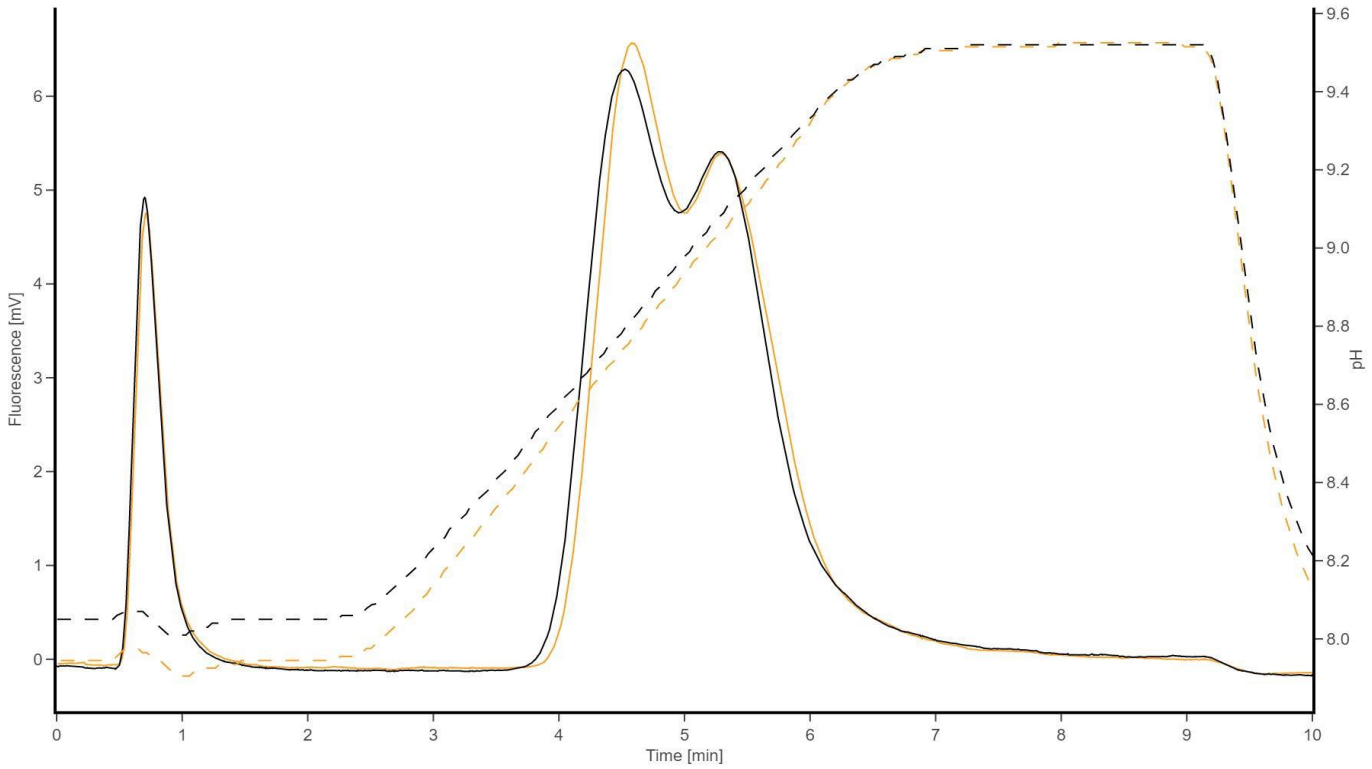
Many Other Parameters Might Influence Empty|Full AAV Capsid Separation

AAV Loading Amount Shifts Elution Time to the Left (Elution at Lower Salt Amount)



Loading (vg/mL column)	Loading (vp/mL column)	Conductivity at the top of full peak [mS/cm]	Conductivity at the top of empty peak [mS/cm]	Resolution of A280 full peak	Shift to the left of the full peak compared to the 1E+13 loading (%)*	Width of A280 full peak (CV)**
1,00E + 11	3.76E+11	15,69	14,02	0,51	-1,8	***
1,00E + 12	3.76E+12	15,58	13,88	0,75	-1,0	***
1,00E + 13	3.76E+13	15,42	13,67	0,89	0	6,6
2,00E +13	7.52E+13	15,39	13,58	0,99	0,2	6,8
4,00 + 13	1.50E+14	15,15	13,32	0,97	1,8	6,5
6,00E + 13	2.26E+14	15,03	13,11	0,95	2,5	6,2
8,00E + 13	3.01E+14	14,87	12,89	0,91	3,6	6,9
1,00E + 14	3.76E+14	14,93	12,92	0,76	3.2	6,0
5,00E + 14	1.88E+15	13,59	10,28	0,45	11,9	10,0

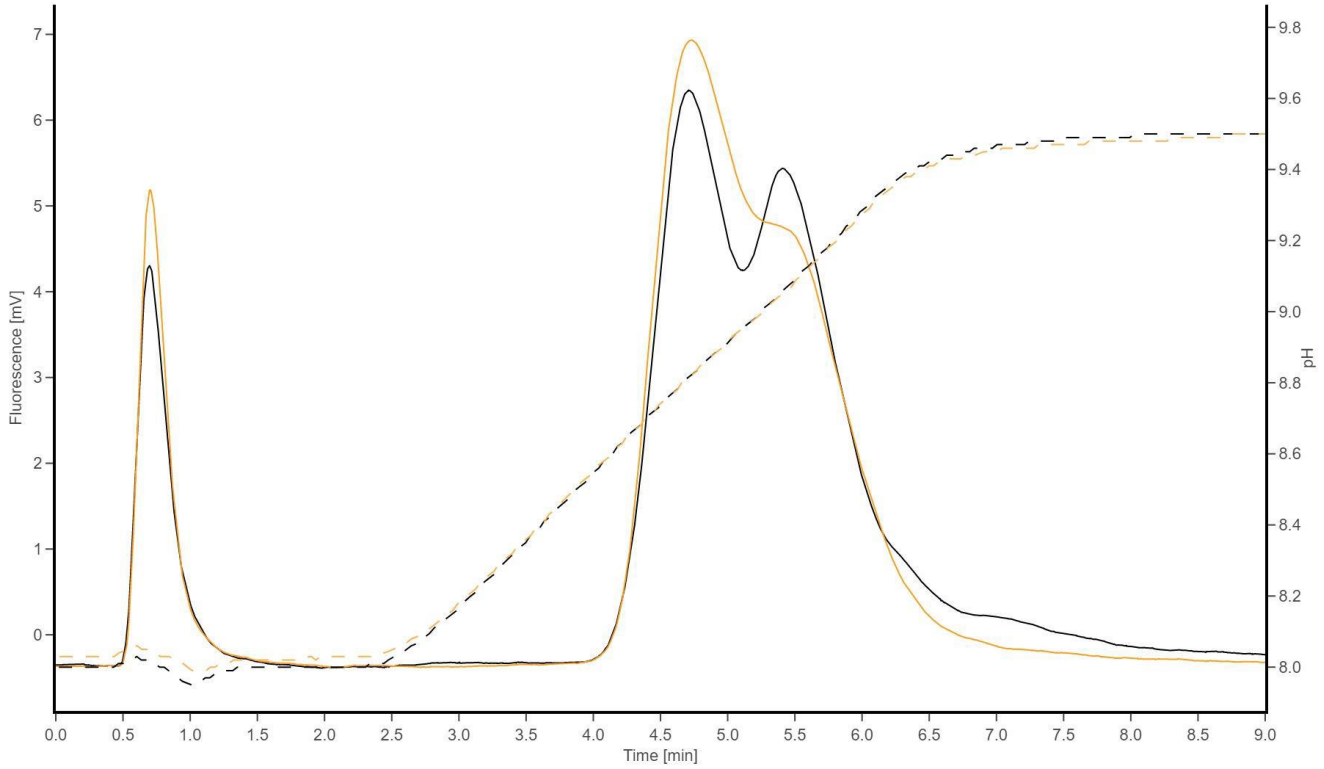
Reproducibility of Buffer Preparation Influence on the Elution Time



Buffer A pH	t_{empty} [min]	R [/]
7,95	4,59	0,55
8,05	4,52	0,58

Chromatograms of Empty|Full AAV separation with buffer A pH 8.00 ±0.05 (CIMmultus PrimaS)

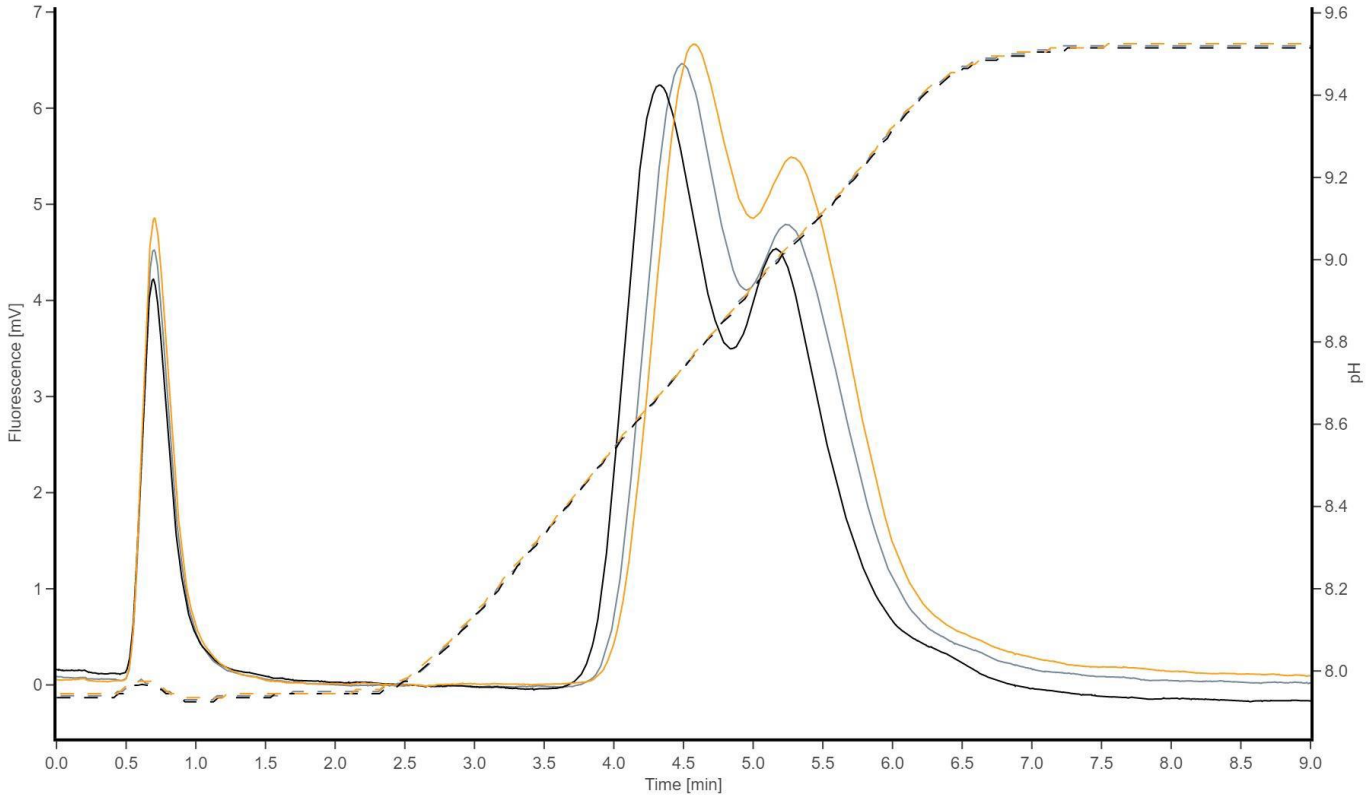
Column Equilibration Influence on the Elution Time



Neutralization after CIP	t_{empty} [min]	R [/]
with	4,71	0,55
without	4,74	0,58

Chromatograms of Empty|Full AAV separation on Specimen unit that underwent different equilibration procedure.

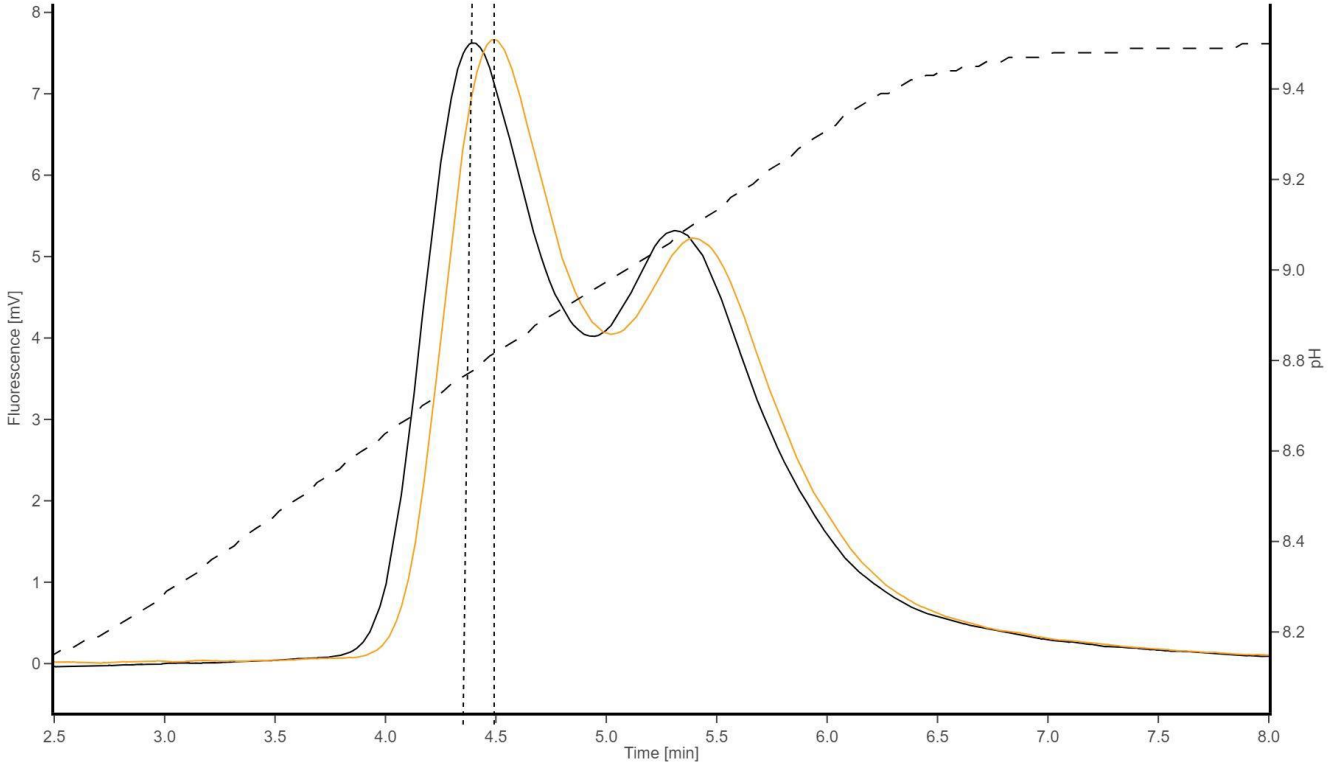
Temperature Influence On The Elution Time



T [°C]	t_{empty} [min]	R [/]
23	4,57	0,54
26	4,49	0,57
30	4,44	0,66

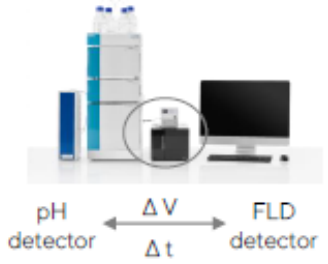
Chromatograms of Empty|Full AAV separation on Specimen unit performed at three
different temperatures 23°C, 26°C and 30°C.

System Void Volume (Time Delay Between Detectors) Influence on the Elution Time

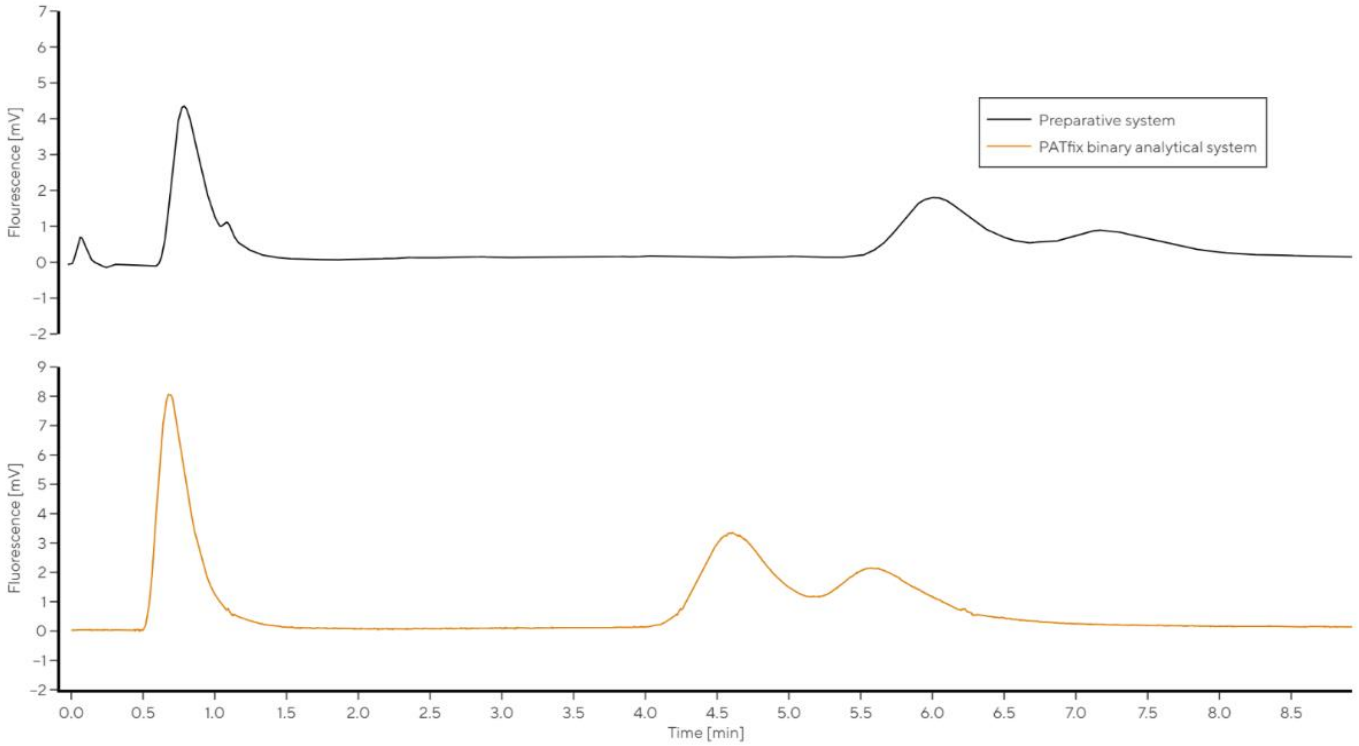


Dt (pH-FLD)	t_{empty} [min]	R [/]
with	4,39	0,64
without	4,49	0,64

Chromatograms of Empty|Full AAV separation with or without FLD detector



Influence of Different Chromatographic Systems - Analytical vs Preparative



Delay corresponds mainly to larger mixing chamber used on preparative system (1.6 mL, whereas 0.25 ml chamber on analytical system).

Conclusions

- On the 25th anniversary Sartorius BIA Separations is introducing the first of it's **new HR product family**, the CIM QA HR.
- The CIM QA HR line features the **very same quaternary amine ligand as well it is produced the same way as the standard CIM QA columns.** Means it can replace existing CIM QA columns straightforwardly.
- **The CIM QA HR acceptance criteria are based on the conductivity values of the empty AAV capsids elution** instead of ion capacity test using small ionic molecules applied for the standard QA columns family.
- New CIM QA empty/full separations methods offer **5-10 times higher resolution** than methods used so far.
- Novel separation methods we **successfully applied at a preparative scale** to enable more robust manufacturing and purer AAV viral vectors.

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Oral Presentations before February 29, 2024

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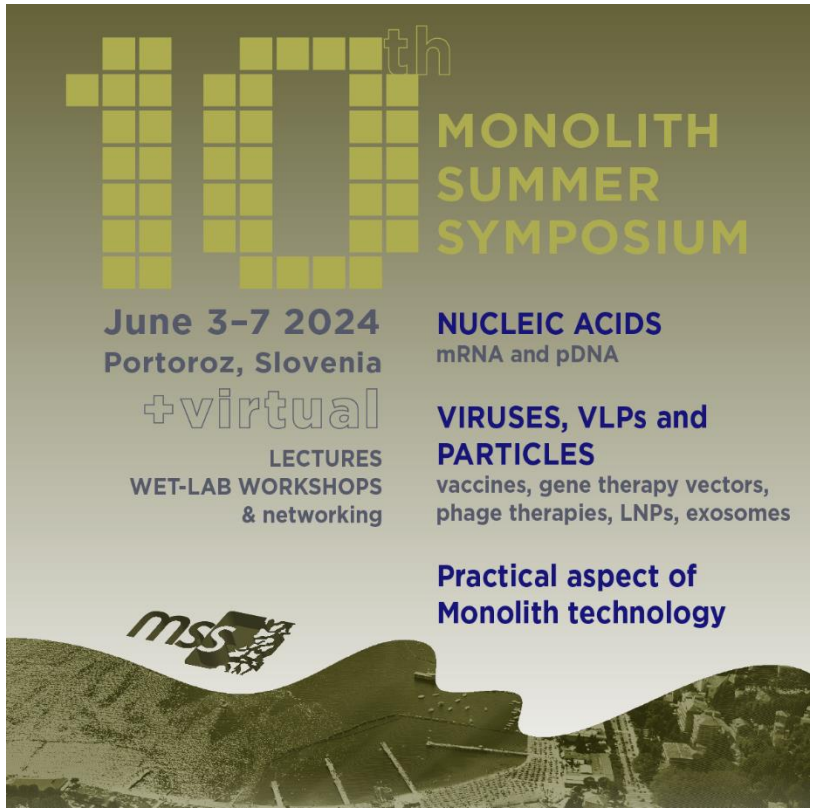

MSS2022 Snapshot

239 Attendees

74 Companies

48 Speakers

Thank you.

Aleš Štrancar
Managing Director at Sartorius BIA Separation
ales.strancar@biaseparations.com

SARTORIUS