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BIA separations

Simplifying Progress

Chromatin (DNA) Removal From Harvest Before an AAV Capture Step Greatly Improves Robustness, Purity, and Yield of the Overall Downstream Process

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ASGCT Exhibitor Showcase, May 13, 2021

About BIA Separations

- The leading developer of monolith technology and the exclusive producer **of CIM® (Convective Interaction Media) monolithic chromatographic columns** for more than 20 years and with > 140 employees currently.
- 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.

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Press Release

Göttingen, November 2, 2020

Sartorius closes acquisition of BIA Separations

- Combination of businesses creates a premium portfolio for advanced therapies
- Course set for successful integration

Testimonials

“We are especially grateful that BIA Separations shared, and operated, with the same sense of urgency we did to help bring gene therapy to the SMA community. BIA’s experience with AAV purification and its chromatographic technology were important contributions and we look forward to our continued work together.”

Andy Stober, Senior Vice President of Technical Operations for AveXis

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Convective Interaction Media (CIM) monolithic columns

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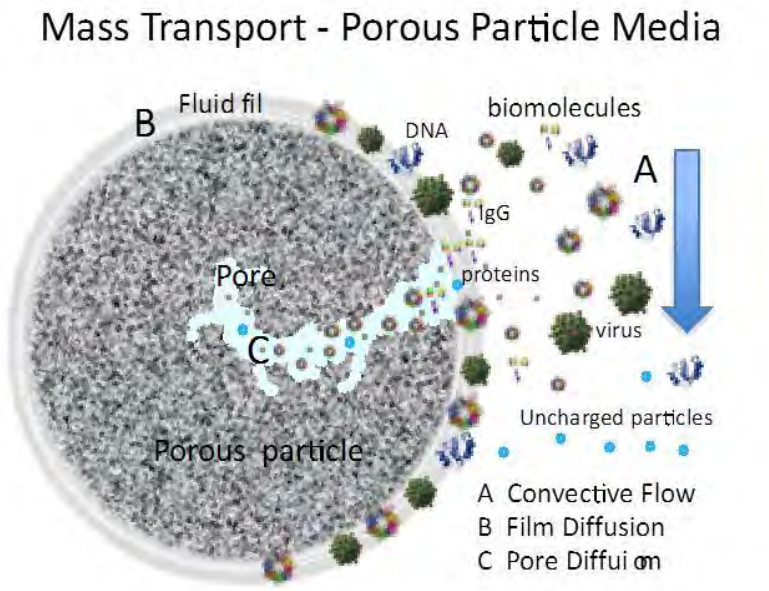
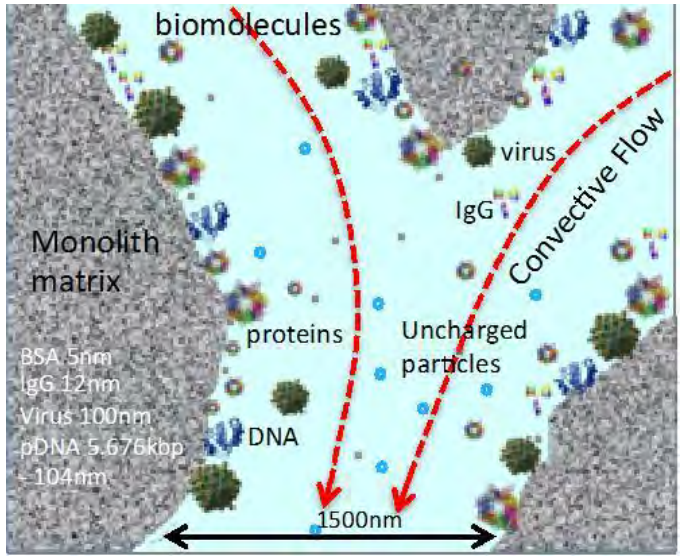
Rapid chromatography processes by convective mass transport





Traditional approach - Porous particle:

- Diffusive mass transport – slow process or **lower resolution**
- Pores too small – very **low capacity for large molecules**
- Counter current flow - shear forces – **lower yields**

Novel UNIQUE approach – Monolithic columns:

- Convective mass transport – **flow independent resolution and capacity**, very fast processes
- Accessible surface for large molecules – **high capacity**
- Laminar flow - No shear forces **– higher yields** of e.g. IgM, Lenti, Adeno, Vaccinia, Flu,...
- Less diffusion and turbulent mixing - **better resolution**

Column body material combines advantages of SS and plastics

- Disposable <u>but multiuse</u>
- Ready-to-use, no packing
- Stainless steel performance characteristics
- cGMP compliant
- allows for robust continuous operations

- Epoxy thermoset composite
- Re-enforced with carbon fibres
- Coated pin-hole free with - USP Class VI Parylene C

BUT:

- <u>3 times cheaper</u>
- <u>5 times lighter</u>

- allow for pre-packed column transport
- customer decides to use disposable column as single or multi use unit

CIMmultus prepacked column product line, 40L available on request

*any intermediate size available if requested number justify the production line costs

Column size/ml*	1	4	8	40	80	400	800	4000	8000
Connector	10-32 coned UNF**			Sanitary TC 1” (25 mm)					
Max pressure, bar	18	20	20	20	20	14	14	7	7
Max flow rate, mL/min	16	50	100	200	400	800	1600	4000	8000
Max flow rate, CV/min	16	12.5	12.5	5	5	2	2	1	1
Bed height, cm	0.6	0.42	0.42	0.95	0.95	2	2	2.2	2.2

**sanitary TC on request

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PATfix™ Fast method development and in-process control HPLC system with unique software

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CIMac analytical columns for PAT HPLC - no carry over of contaminants or viruses

Available:

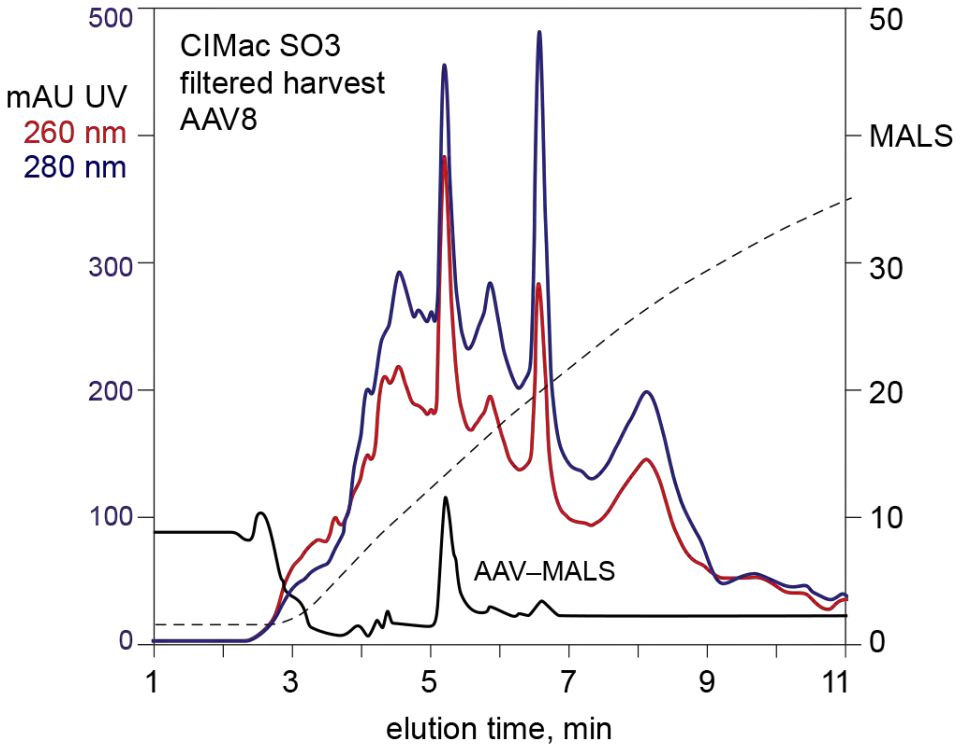
- CIMac™ QA
- CIMac™ DEAE
- CIMac™ SO3
- CIMac™ EDA
- CIMac™ pDNA
- CIMac™ Adeno
- CIMac™ AAV empty/full

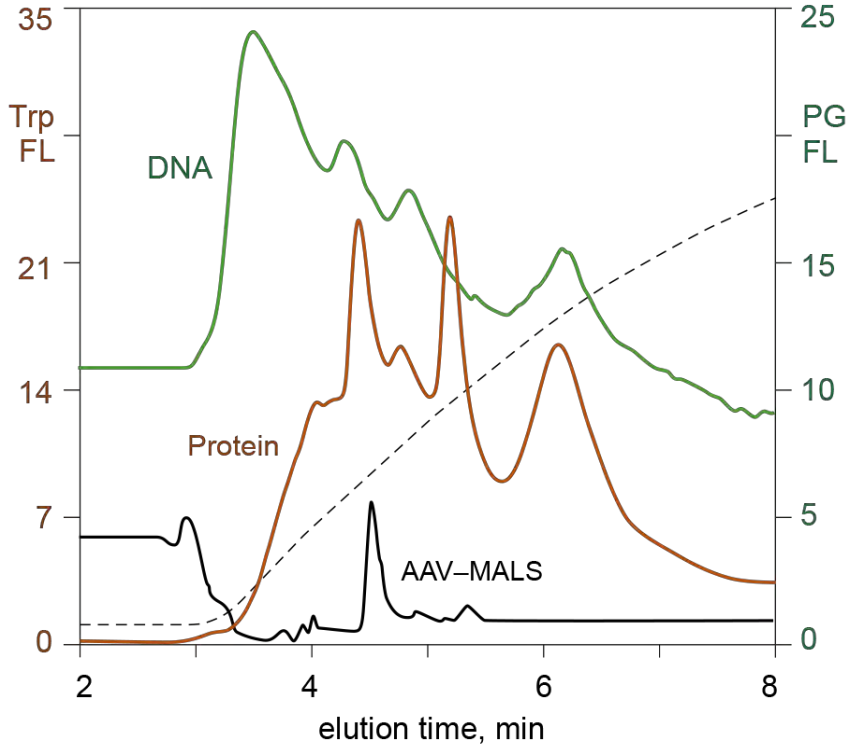
Soon to come:

- CIMac™ AAV total
- CIMac™ Lenti
- CIMac™ Vaccinia

To enable fast process development high performance analytics is mandatory

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



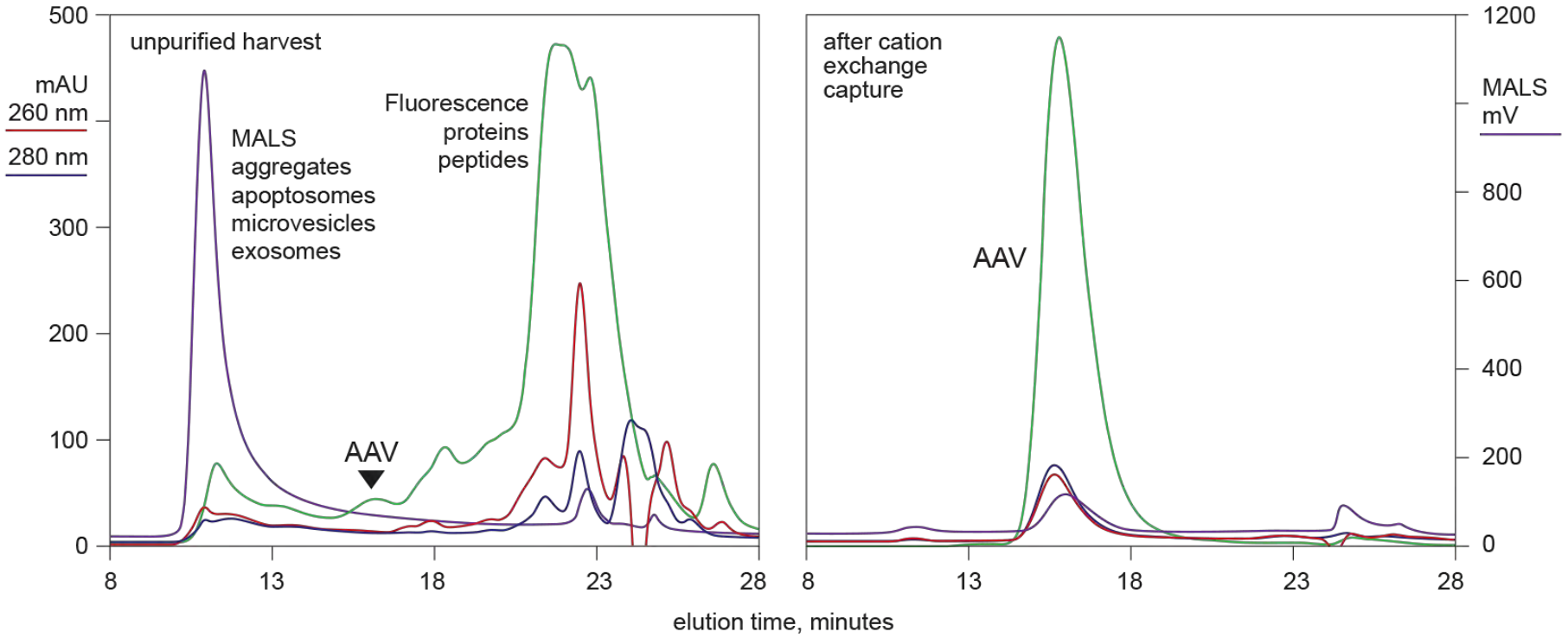


PATfix and CIMac ➔
Process understanding

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**.

When working with complex samples orthogonal analytical methods should be used to prevent mistakes – SEC method orthogonal to IEX

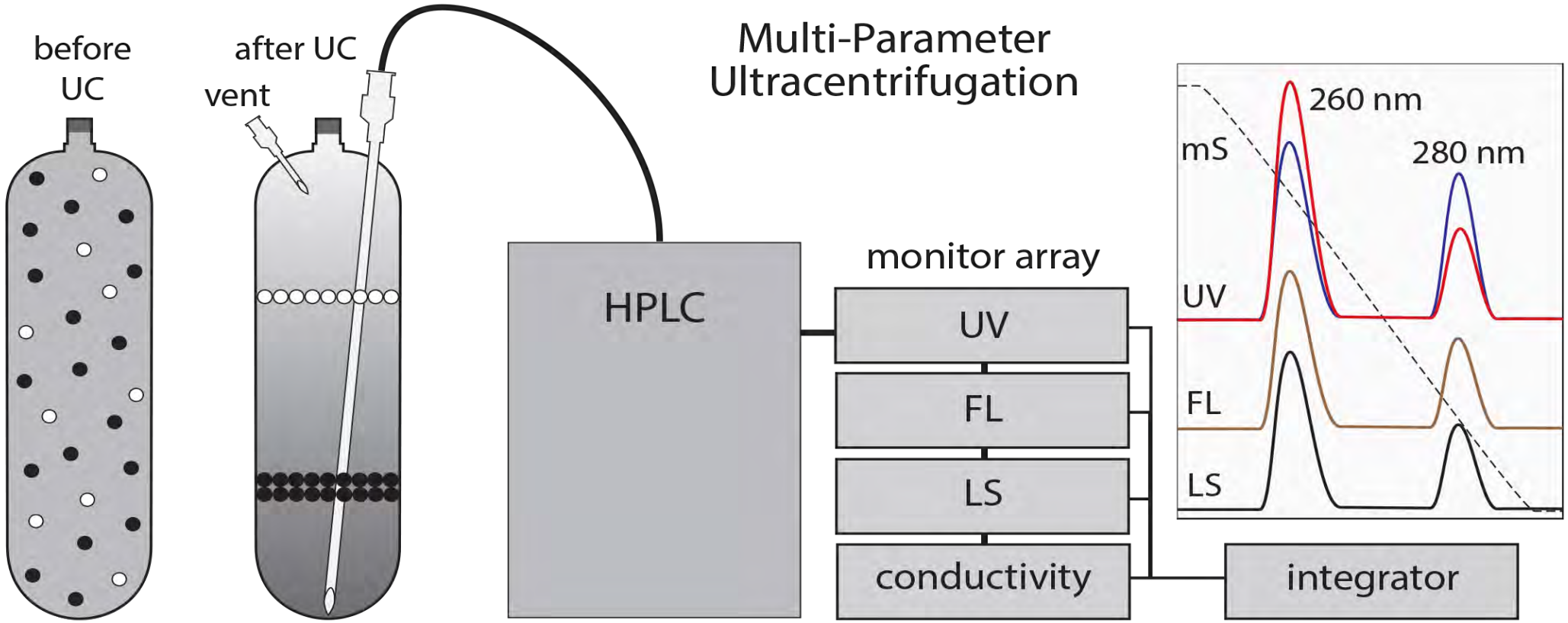
Analytical SEC. TSKgel™ G4000SWxl. 0.5 mL/min.



Fluorescence enables direct detection of virus in harvest. MALS highlights large contaminants at early process stages and confirms the identity of AAV at later process stages.

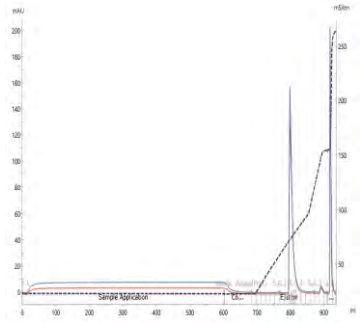
Centrifugram – analysis of ultracentrifuge fractions by HPLC detectors

Density gradient fractionation followed by stratigraphic analysis through an HPLC detector array.



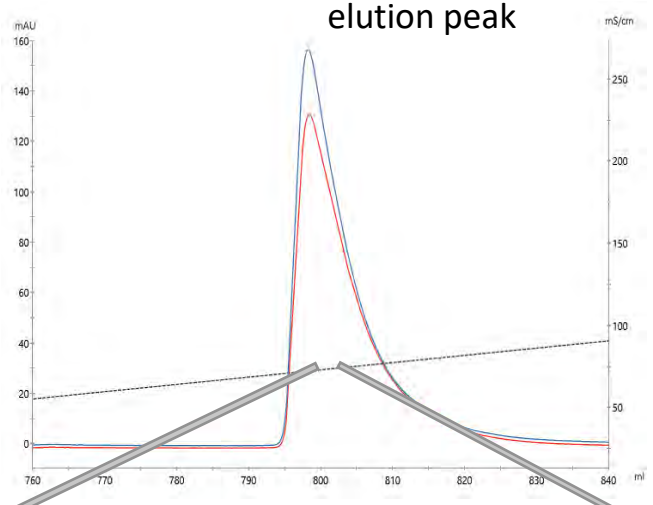
Orthogonal tools to study AAV particles distribution – HPLC : ultracentrifuge

Filtered lysate AAV2/8 from Sf9; TFF/Kryptonase™ treatment



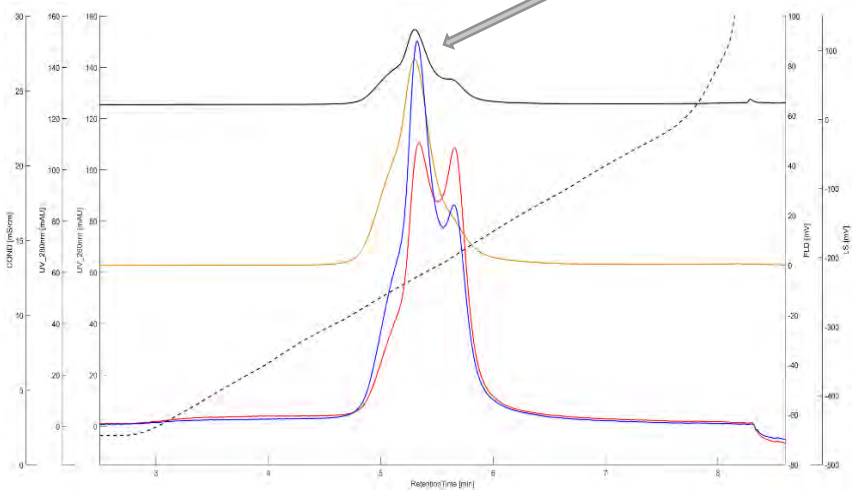
Zoom-in

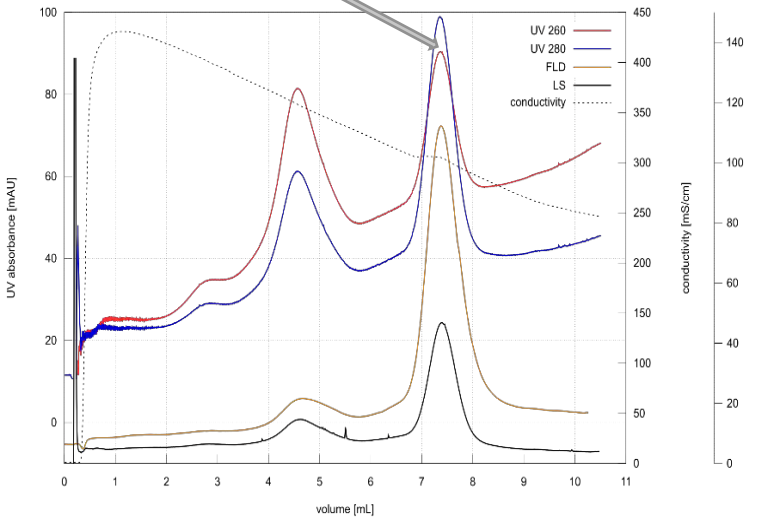
cation exchange elution peak



Detectors:
Red – UV 260 nm
Blue – UV 280 nm
Orange – tryptophan fluorescence
Black - MALS

Chromatogram: HPLC with 3 detectors using QA column – faster gradient





Centrifugram: CsCl ultracentrifuge with 3 detectors – **faster than AUC, less sample needed**

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AAV Manufacturing Platform Process Development – towards ultra low residual DNA/RNA

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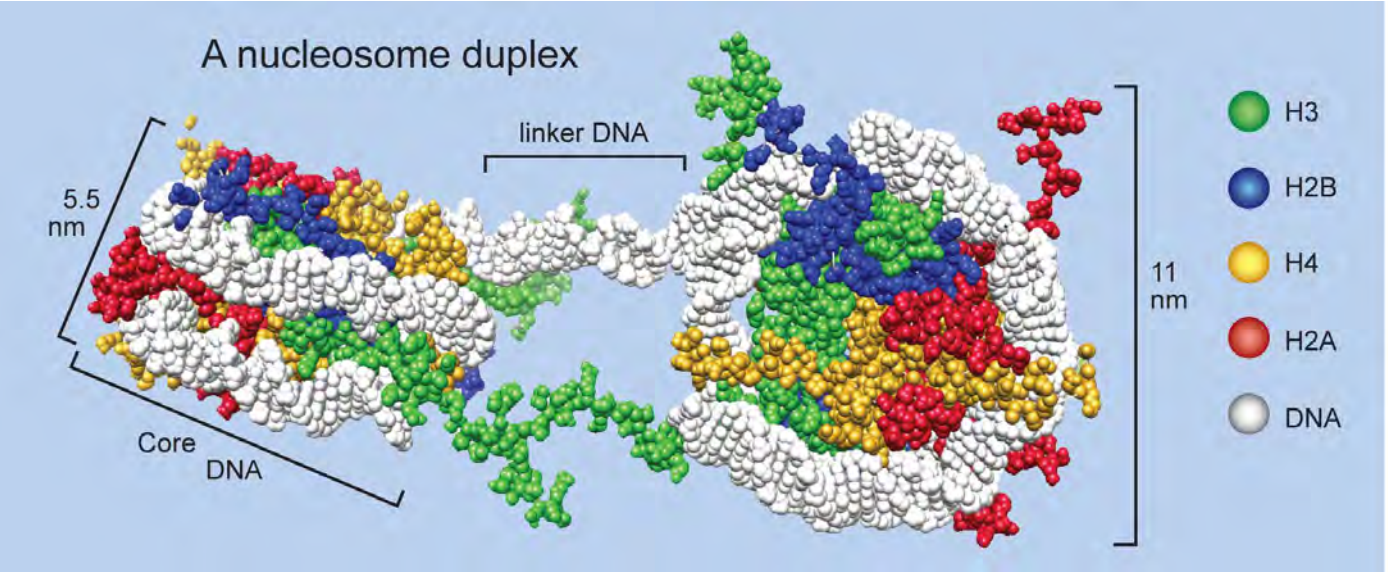
Intracellular produced AAV - after cell lysis sample is reach with Chromatin

For robust process and to reach ultra low residual DNA/RNA proper management of Chromatin structures is mandatory.

The basic structural subunit of chromatin is a nucleosome.

It consists of a histone octamer wrapped with 1.6 turns of DNA; about 150 bp.

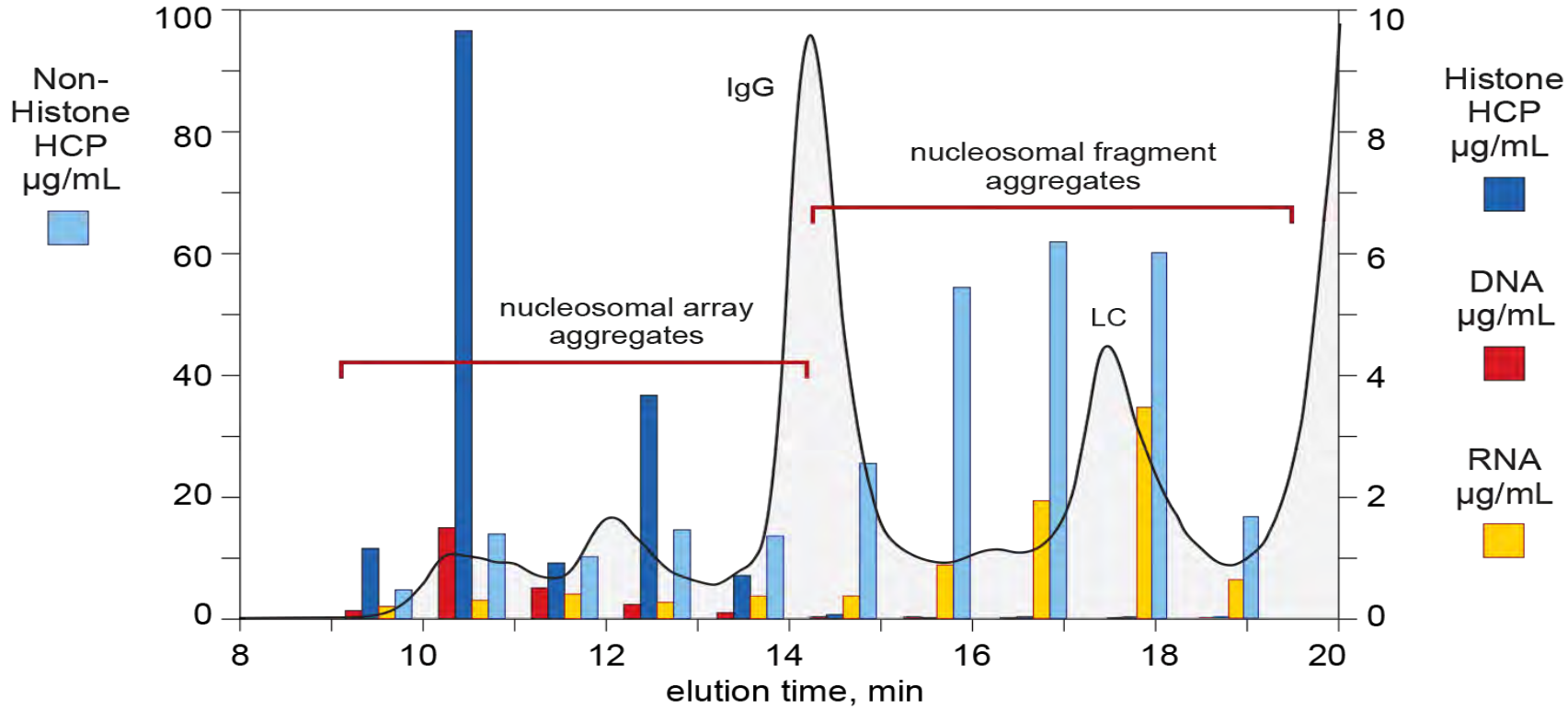


- **Histones** are extremely hydrophobic and highly positively charged, with isoelectric points ranging from 9 to 11. DNA has a pK of about 2.6.

- The net charge of **chromatin** is roughly neutral but its exposed components still retain their extreme charge characteristics. Both also participate in metal affinity, hydrogen bonding, and van der Waals interactions.

Chromatin from the CHO cell lysate – relatively low amount compared to the IgG

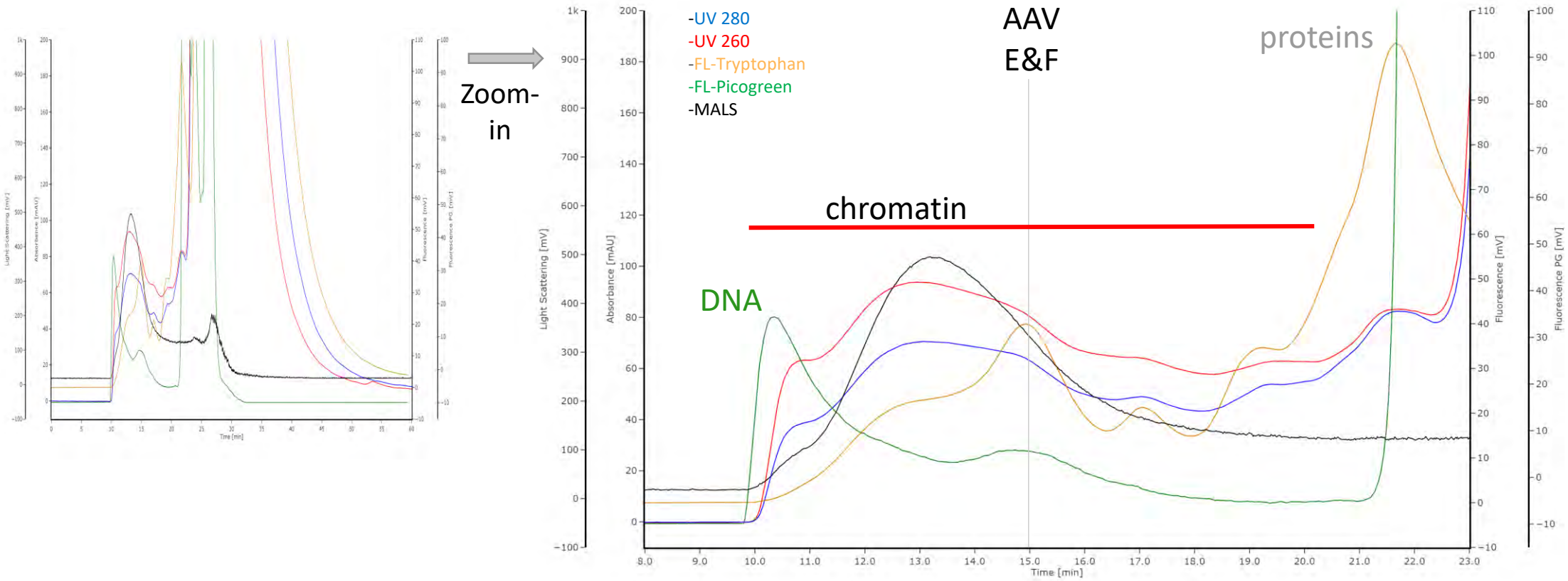
Filtered CHO Harvest containing prospective biosimilar Herceptin™
Analytical SEC. Host DNA by ddPCR. Histones and other HCP by ELISA.



Chromatin aggregates built on nucleosomal arrays range in size from 10–400 nm. Aggregates based on nucleosomal fragments range from 2–10 nm. Arrays and fragments both act as nucleation centers for accretion of non-nucleosomal proteins and RNA. Note the different scale for non-histone HCP.

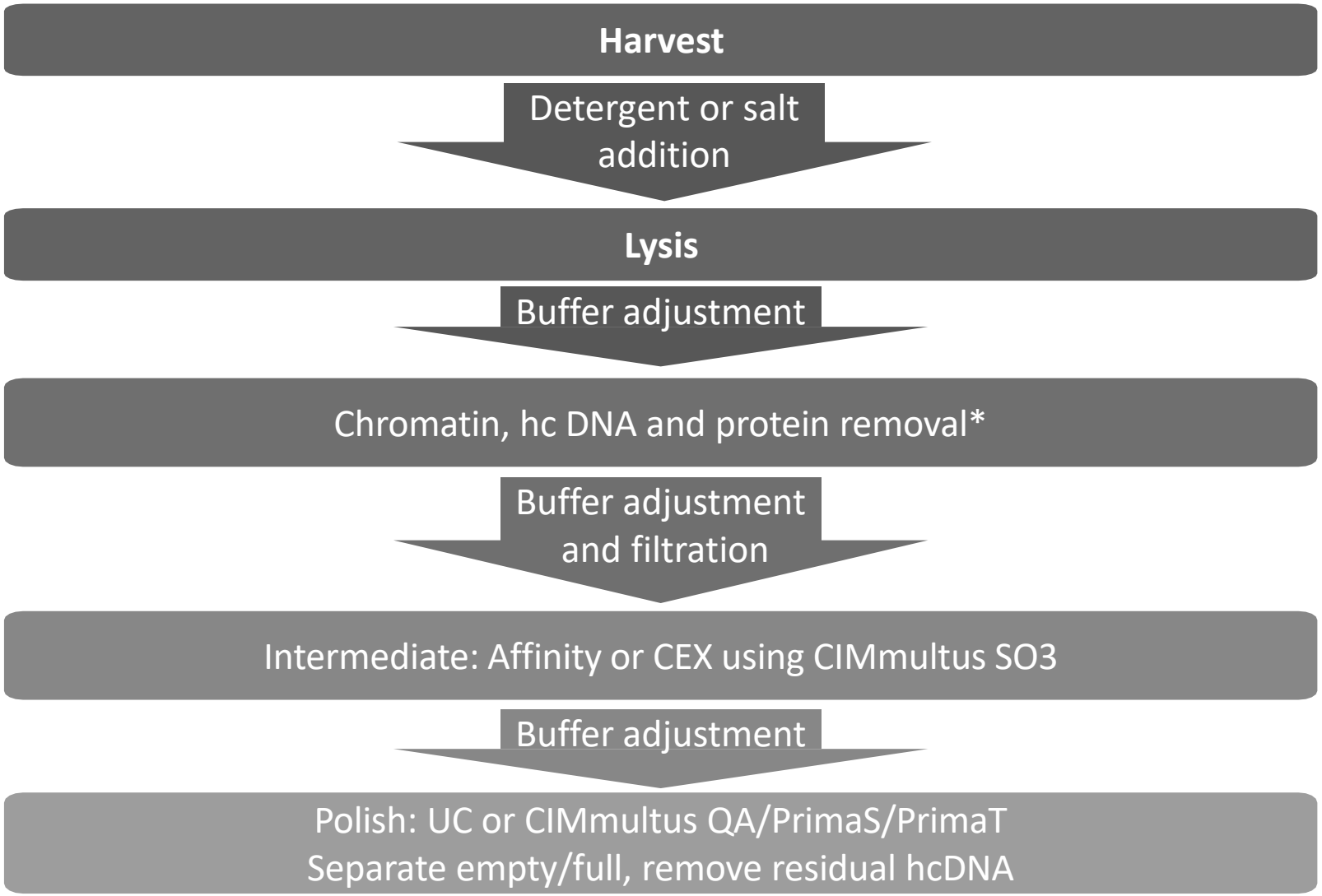
Low AAV concentration (compared to IgG) inflates the Chromatin-to-product ratio

PATfix HPLC with multiple detectors – very powerful tool for Chromatin detection



Analytical HPLC SEC: TSKgel™ G4000SWxl. 0.5 mL/min. Sample prestained with Picogreen™. Intrinsic tryptophan fluorescence amplifies sensitivity for proteins about 20-fold over UV and enables direct visualization of the AAV.

Chromatin removal options



***Multiple options, alone or combined:**

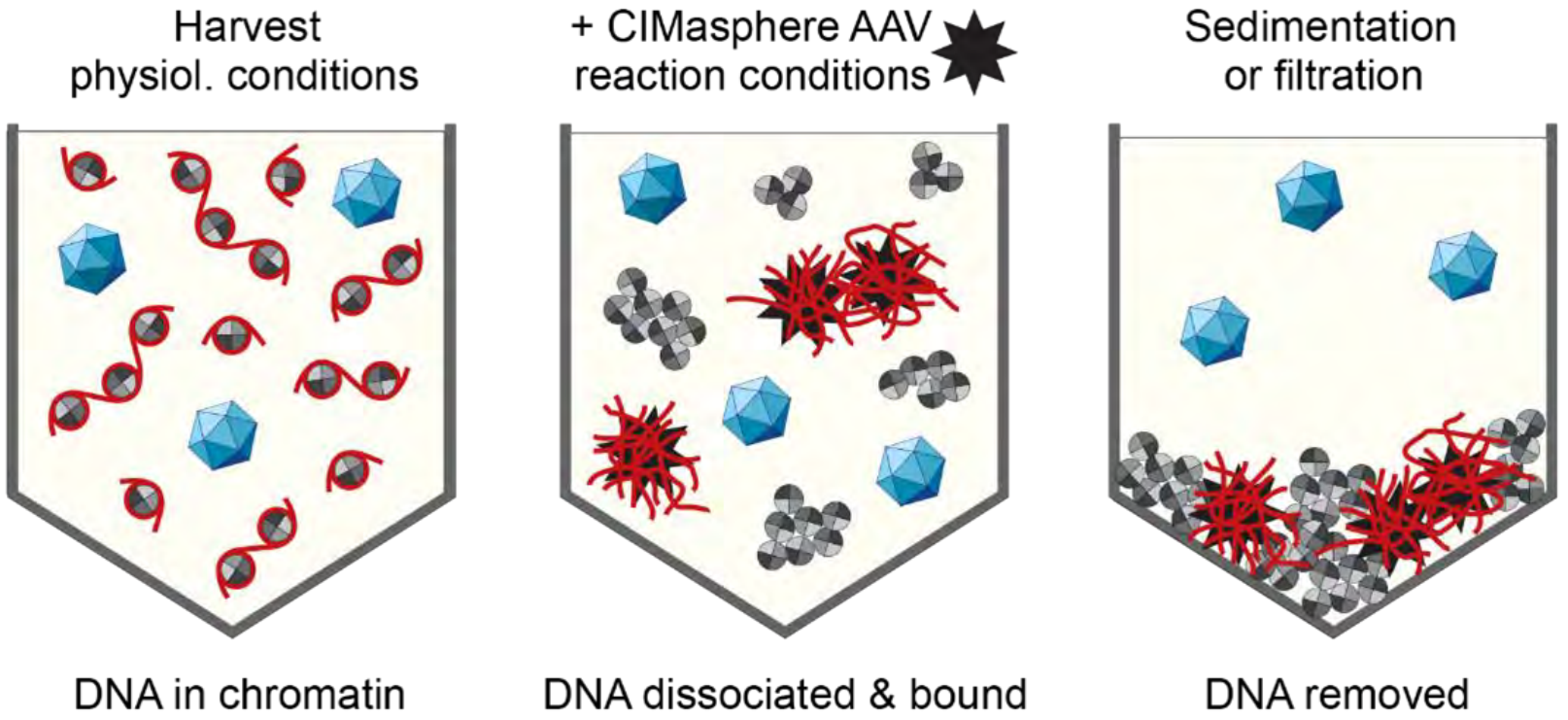
- Flocculation/DNAse treatment (Y/N)
- TFF/DNAse treatment (Y/N)
- **Solid phase extraction/DNAse treatment (Y/N)**
- DNAse treatment (Y/N) followed by acidic precipitation and direct load on the SO3 column

Example: Chromatin Solid phase extraction (SPE) using particles

CIMasphere™ AAV-extracted from 100 mL SF9 lysate, AAV2/8

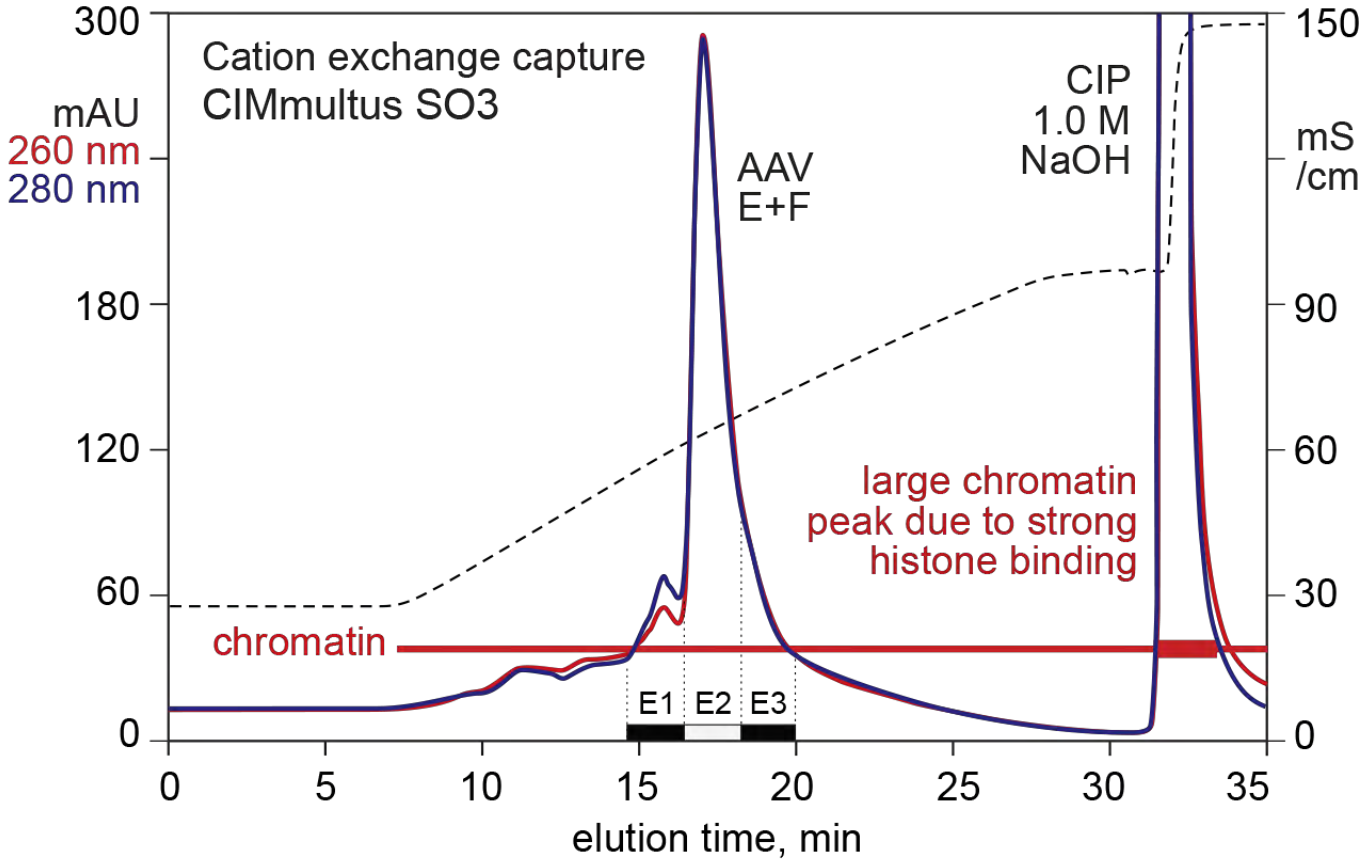
CIMasphere is a particulate solid phase that binds Chromatin/DNA so strongly it becomes dissociated from its pre-existing associations.

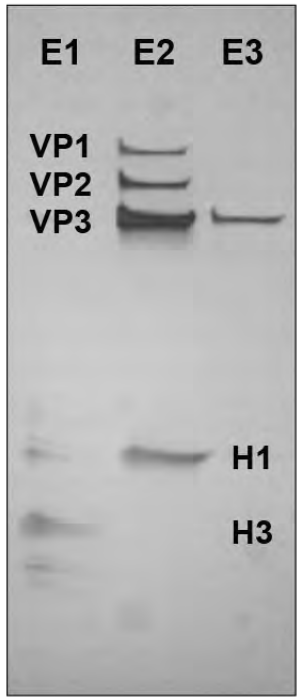
After incubation the particles are removed by any convenient method; centrifugation, membrane or depth filtration.



Post SPE capture of the AAV by cation exchange chromatography

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 that DNA enables better separation of empty and full capsids.**



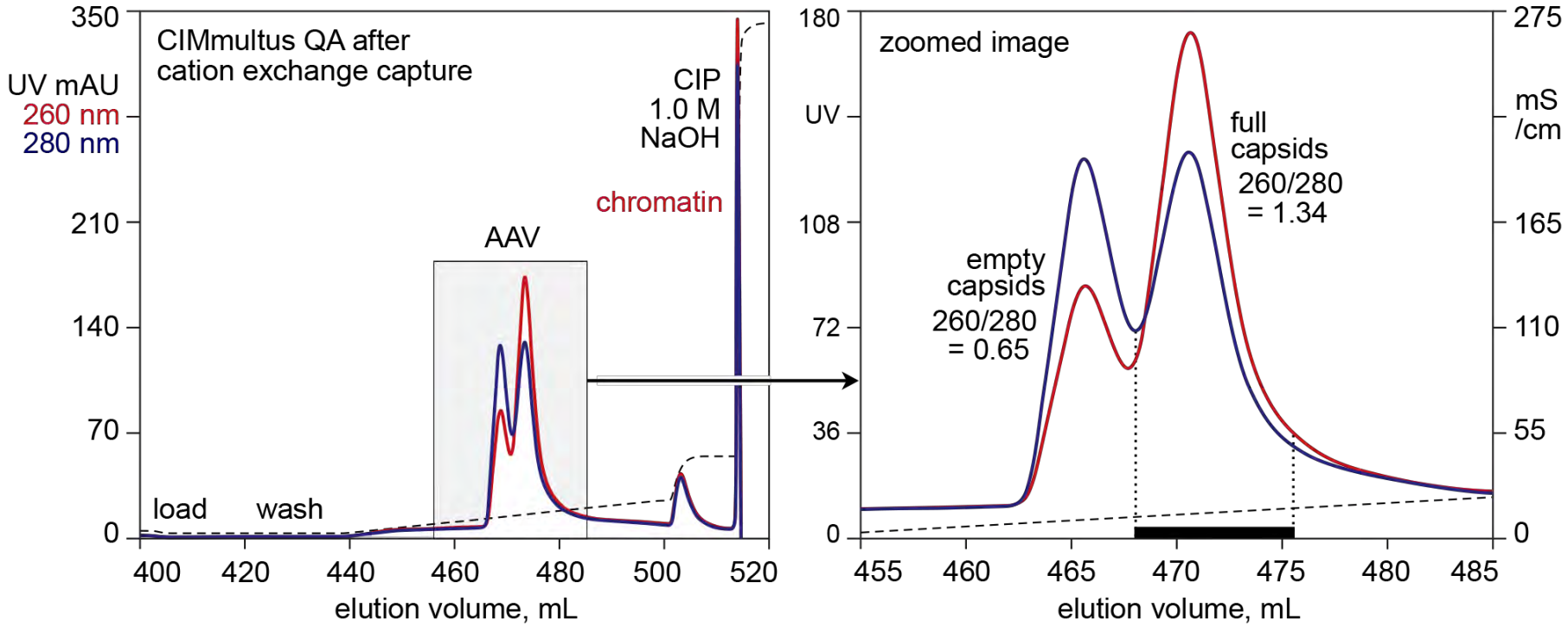


CIMmultus™ SO3, 1 mL, 2 µm channels, 10 CV/min

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

AAV Empty/Full separation AAV by anion exchange chromatography with Mg^{2+}

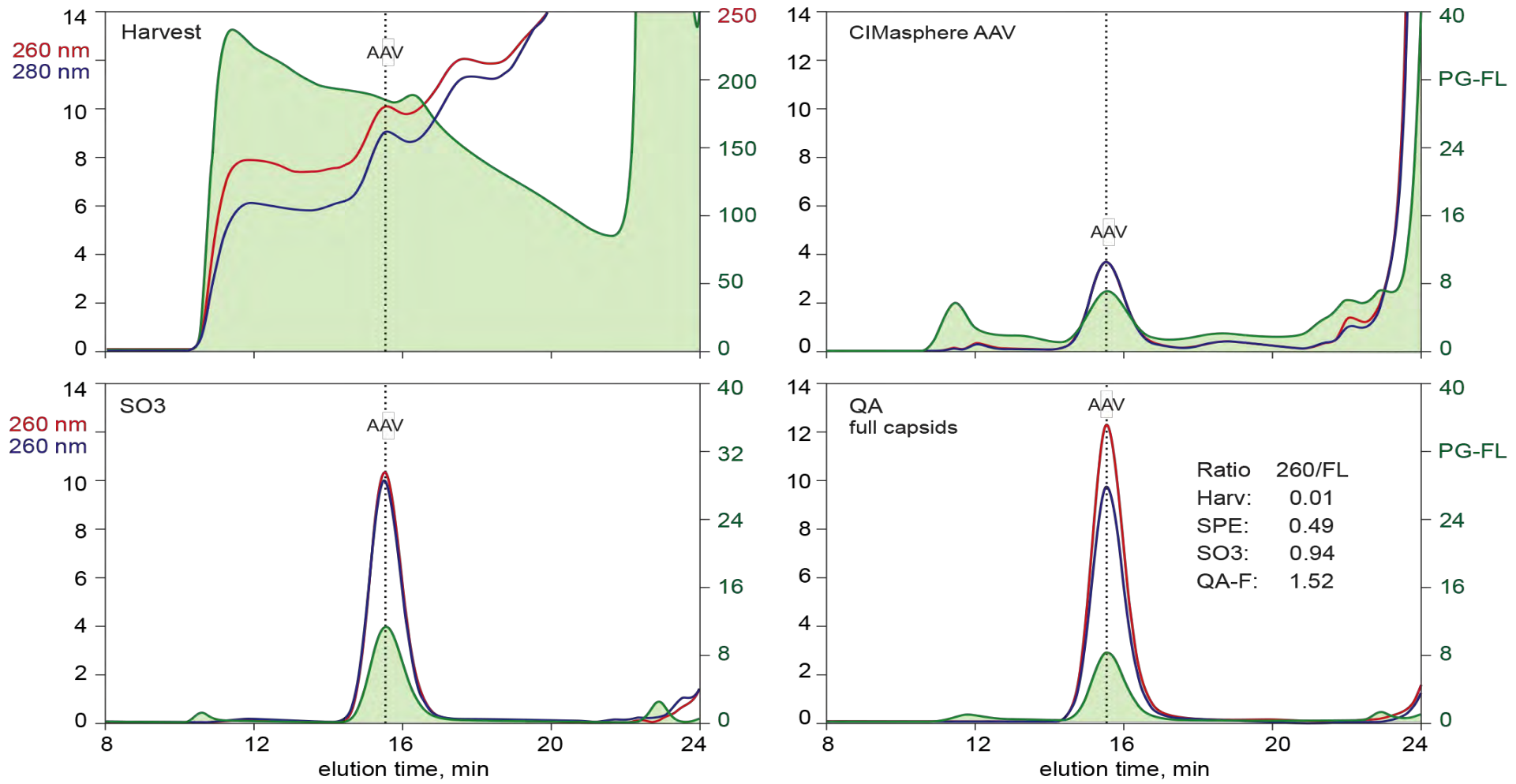
CIMmultus SO3 pre-purified sample loaded on the QA column.



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.**

Monitoring DNA reduction across process steps

Samples prestained with Picogreen, analysed by HPLC SEC with UV and FLD. TSKgel™ G4000SWxl. 0.5 mL/min



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Ways to improve elimination of empty, full and damaged capsids

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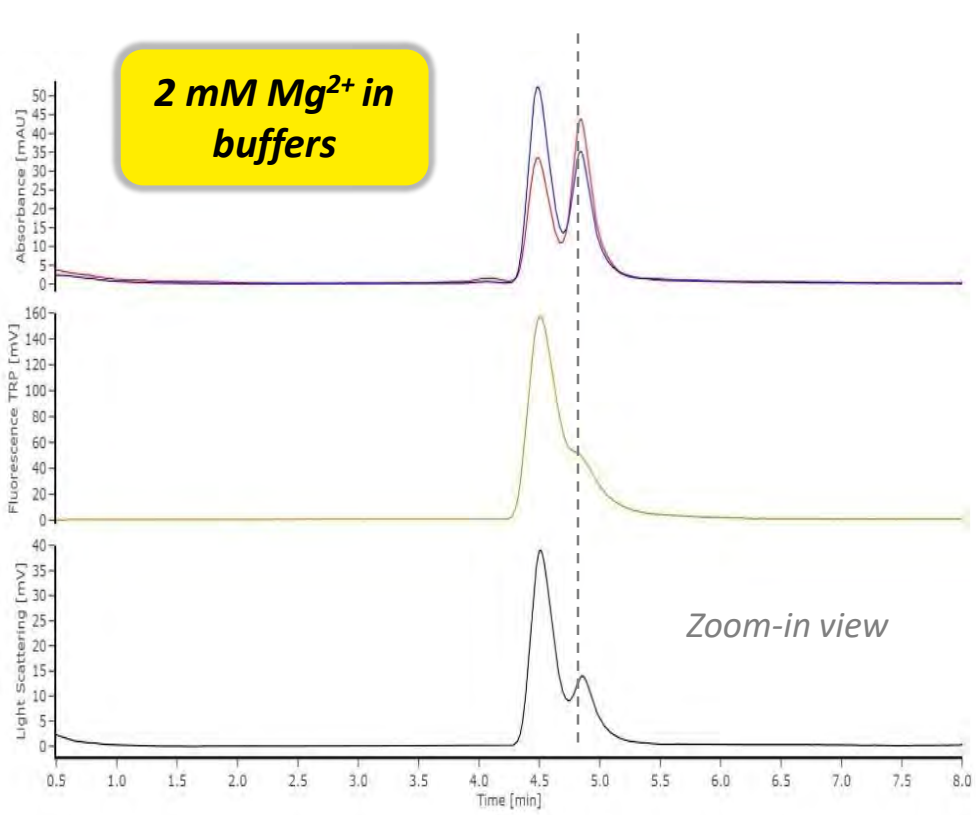
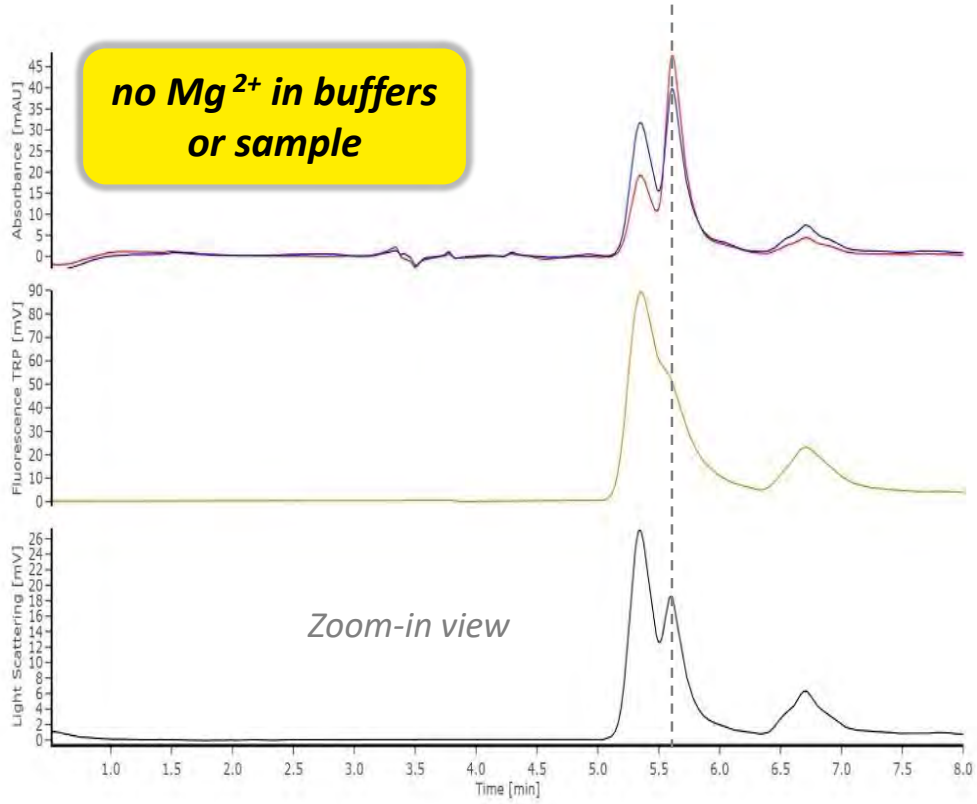
Influence of Mg^{2+} on separation of AAV capsids using CIM QA column

Sample: AAVrh10 pre-purified using CIM SO_3 column





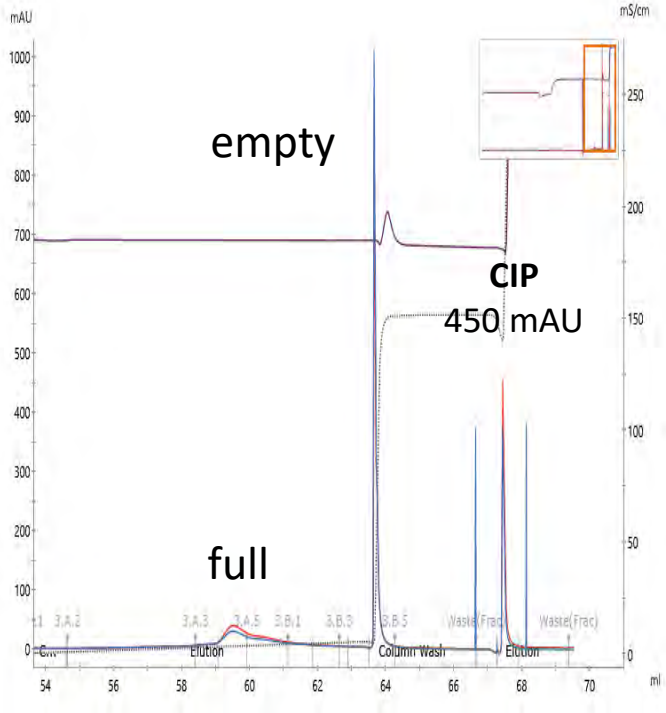
Detectors:
Red – UV 260 nm
Blue – UV 280 nm
Orange – tryptophan fluorescence
Black - MALS

Column: CIMac™ QA (Pores 1.3 µm); Buffer A: 20 mM BTP, 1% Sorbitol, 0.1% Poloxamer, pH 9.5 (+ 2 mM $MgCl_2$ for buffer with Mg^{2+}); Buffer B: Buffer A + 400 mM NaCl (+ 2 mM $MgCl_2$ for buffer with Mg^{2+}); Gradient: 0 % B to 100 % B in 100 CVs; Flow rate: 10 CV/min

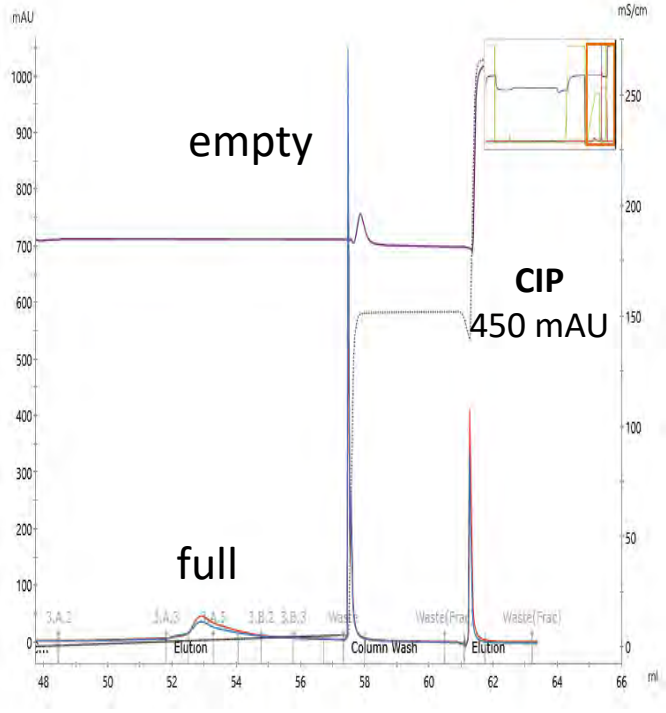
Influence of charging PrimaT with different metal ions on E/F separation

Filtered lysate **AAV2/8** from Sf9; TFF/Kryptonase treatment, SO3 purified

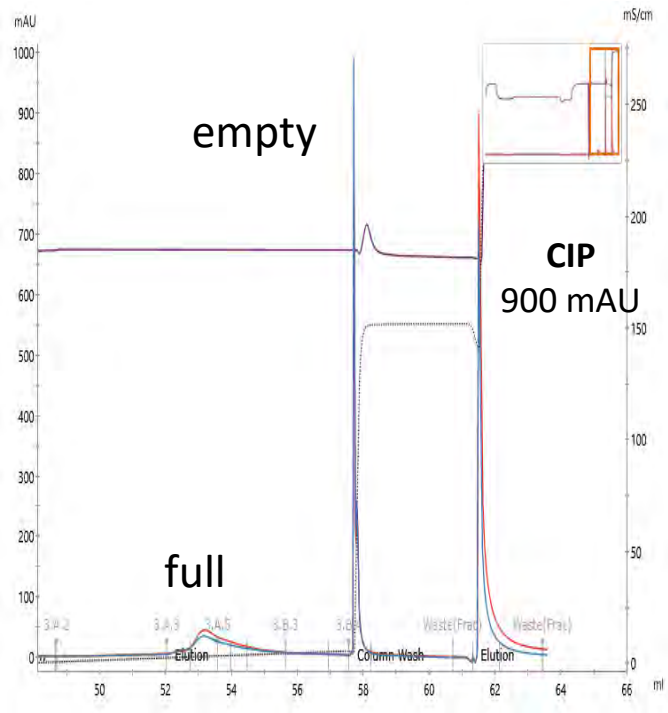
PrimaT - no metal



PrimaT - Mg



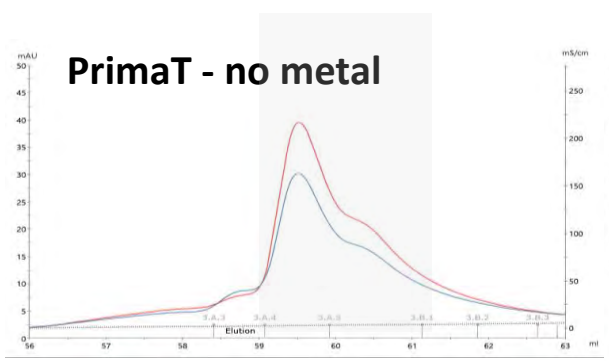
PrimaT - Fe



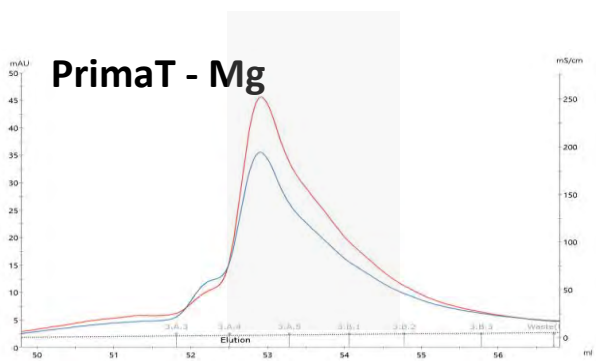
Column: CIM PrimaT (2 µm ID channels); Buffer A: 50 mM TRIS, 12 mM boric acid, 1% saccharose, 0.1% Poloxamer, pH 9.0; Buffer B: Buffer A + **50 mM $MgCl_2$**; Gradient: 0 % B to 50 % B in 50 CVs; **Flow rate: 10 CV/min**

Density gradient ultracentrifugation to check the purity of full capsids

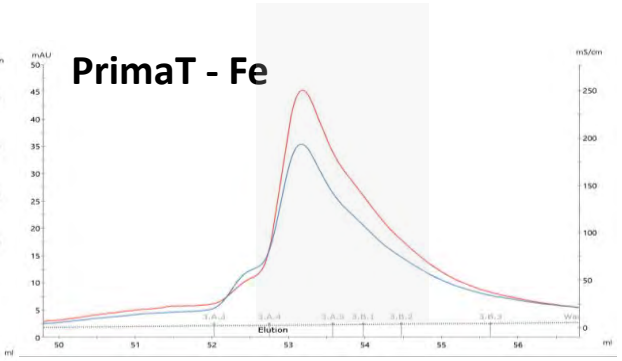
HPLC:



Zoom-in (elution)



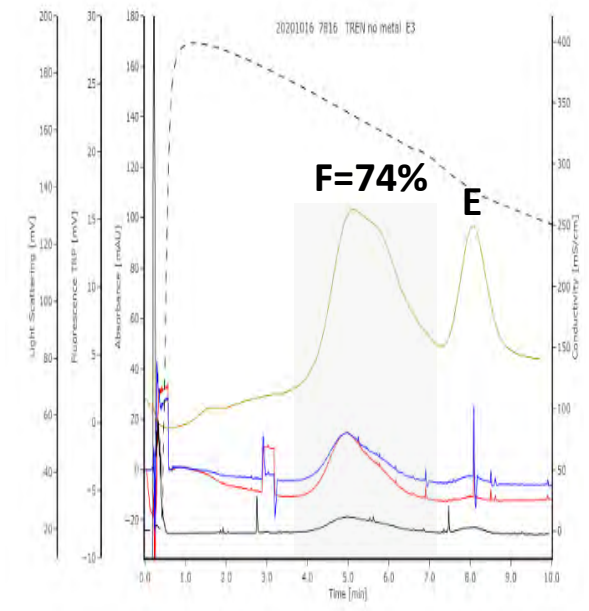
Zoom-in (elution)

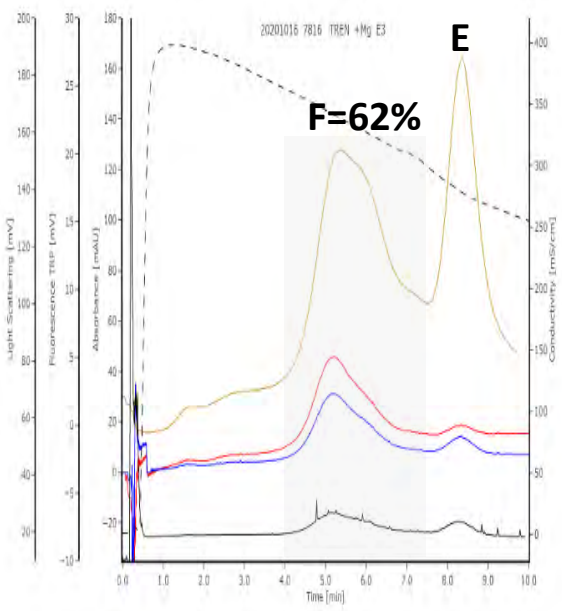


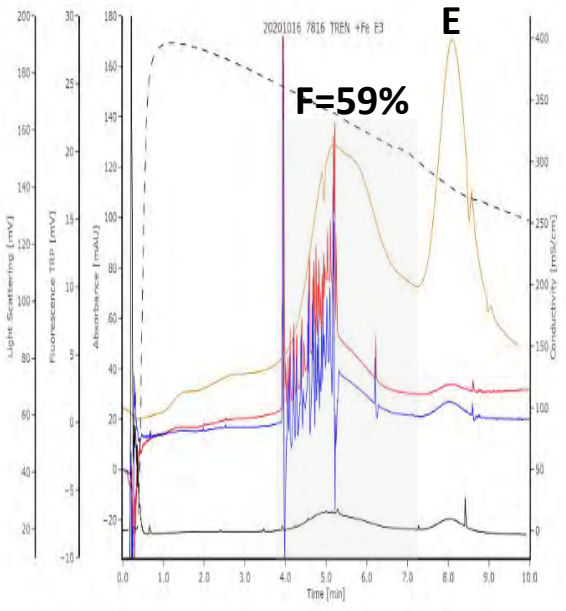
Zoom-in (elution)

Detectors:
Red – UV 260 nm
Blue – UV 280 nm
Orange – tryptophan fluorescence
Black - MALS

UC:







Further investigations needed to understand heterogeneity of the full peak.

CIM PrimaT - yield and purity

New E/F no metal		VECTOR GENOME (ddPCR)		
		date:	14.10.2020	
Fraction	Volume (ml)	vector genome concentration (vg/ml)	vector genome in fraction (vg)	% of full capsids
CEX-44 E2				
LOAD	9,00	7,1E+10	6,4E+11	100%
New E/F no metal				
E2 (empty)	0,68	8,3E+09	5,6E+09	1%
E3 (full)	2,11	1,9E+11	3,9E+11	61%
E4 (tail)	1,81	3,4E+10	6,2E+10	10%
E5 (empty)	0,78	8,4E+10	6,5E+10	10%
CIP	0,99	under limit of det.		
		SUM recovery total		**82%**
		SUM recovery fraction		**71%**

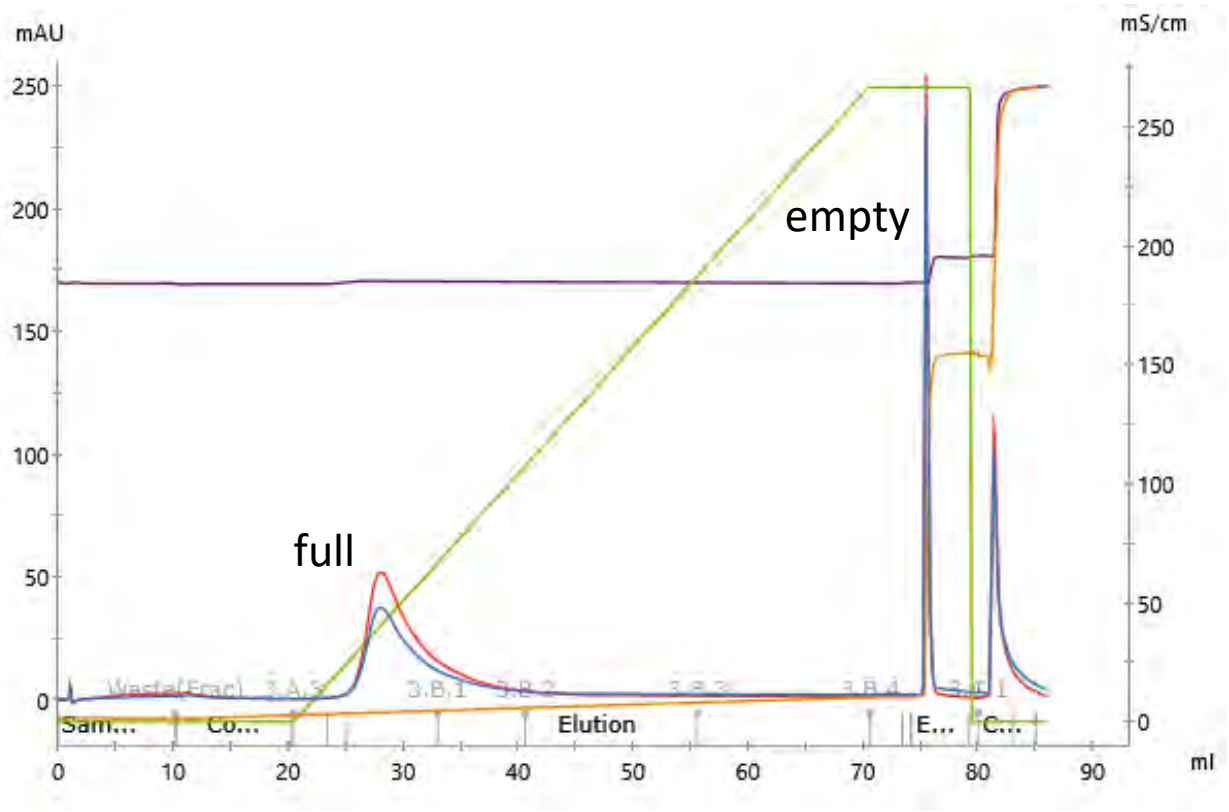
New E/F + Mg		VECTOR GENOME (ddPCR)		
		date:	14.10.2020	
Fraction	Volume (ml)	vector genome concentration (vg/ml)	vector genome in fraction (vg)	% of full capsids
CEX-44 E2				
LOAD	9,00	7,1E+10	6,4E+11	100%
New E/F + Mg				
E2 (empty)	0,71	9,9E+09	7,0E+09	1%
E3 (full)	1,59	2,6E+11	4,2E+11	66%
E4 (tail)	2,73	4,4E+10	1,2E+11	19%
E5 (empty)	0,67	5,1E+10	3,4E+10	5%
CIP	1,34	under limit of det.		
		SUM recovery total		**91%**
		SUM recovery fraction		**84%**

New E/F + Fe		VECTOR GENOME (ddPCR)		
		date:	15.10.2020	
Fraction	Volume (ml)	vector genome concentration (vg/ml)	vector genome in fraction (vg)	% of full capsids
CEX-44 E2				
LOAD	9,00	7,1E+10	6,4E+11	100%
New E/F + Fe				
E2 (empty)	0,74	9,9E+09	7,3E+09	1%
E3 (full)	2,18	2,3E+11	5,1E+11	79%
E4 (tail)	2,53	4,0E+10	1,0E+11	16%
E5 (empty)	0,69	5,2E+10	3,6E+10	6%
CIP	1,81	under limit of det.		
		SUM recovery total		**102%**
		SUM recovery fraction		**95%**

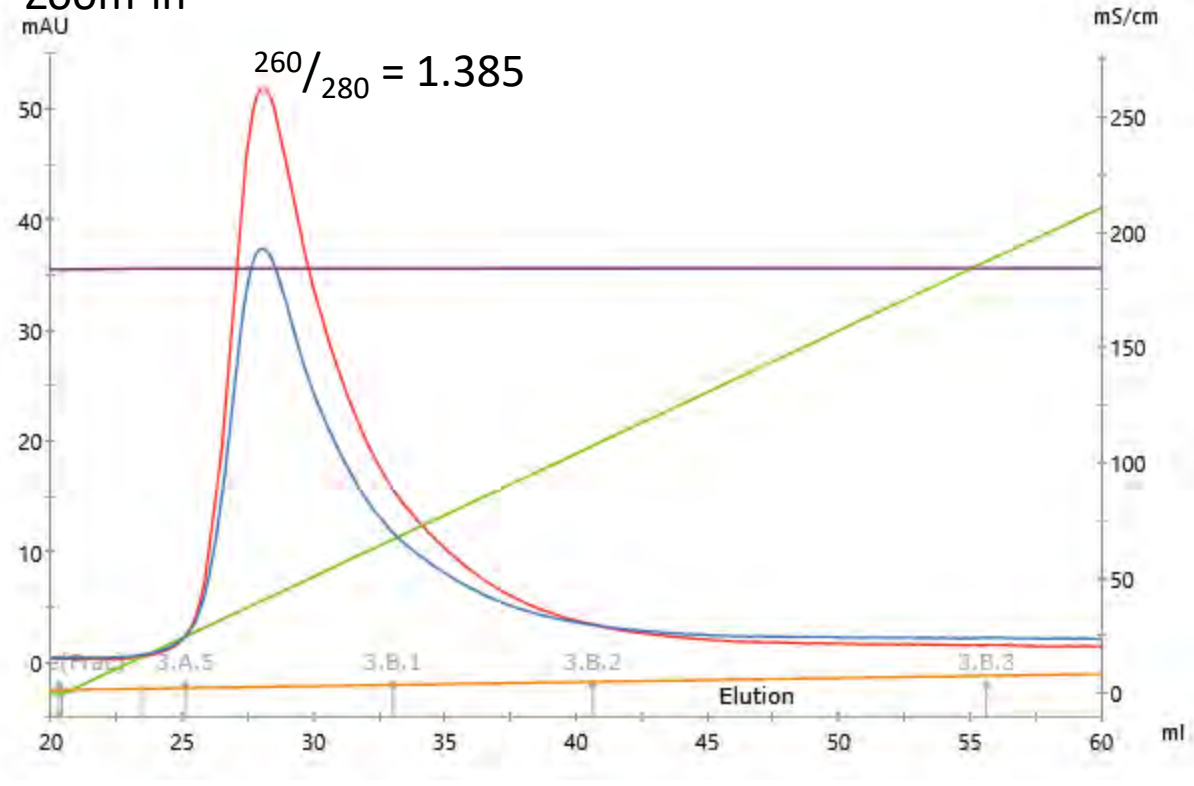
	V (fraction) [ml]	**c (total DNA) [ng/ml]**	**c (total DNA in CIP) [ng]**
New E/F no metal_CIP	0,99	4,02	3,98
New E/F + Mg_CIP	1,34	27,31	**36,60**
New E/F + Fe_CIP	1,81	19,82	**35,87**

PrimaT works for different serotypes - purification of AAV9 full capsids

AAV9 clarified harvest from Sf9 cells was concentrated and purified using combination of tangential flow filtration, nuclease treatment and cation exchange capture. Easy conversion to step gradient format.



Zoom-in

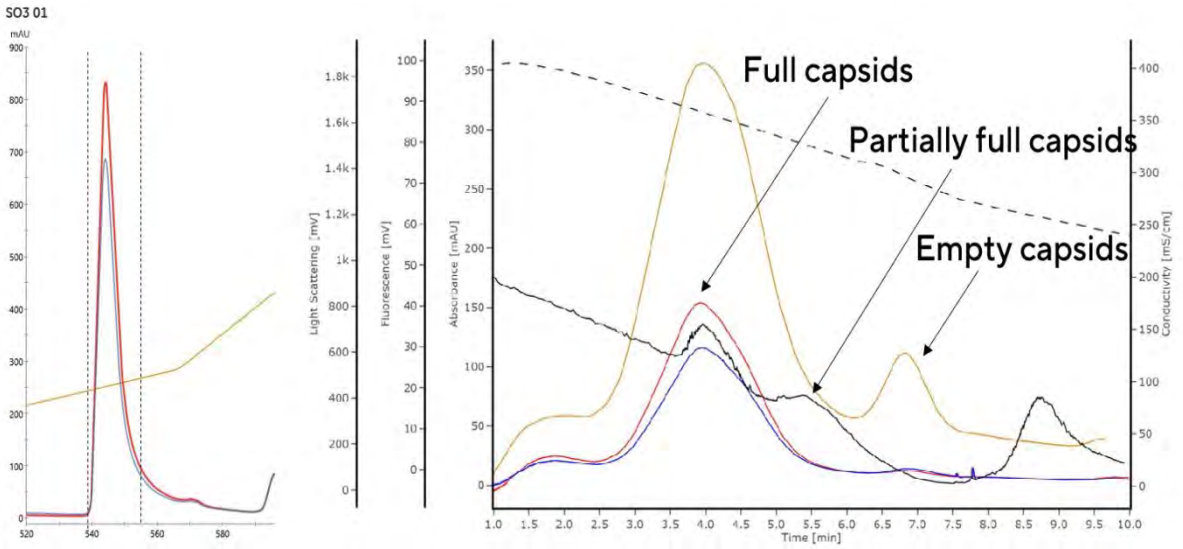


CIM PrimaT column (2 µm ID channels); Buffer A: 20 mM BTP, 2 mM MgCl2, 1 % saccharose, 0.1 % poloxamer, pH 9.0; Buffer B = Buffer A plus 50 mM MgCl2; **Empty capsid wash: Buffer A with 2 M NaCl**

AAV9 full capsid purity check using ultracentrifuge

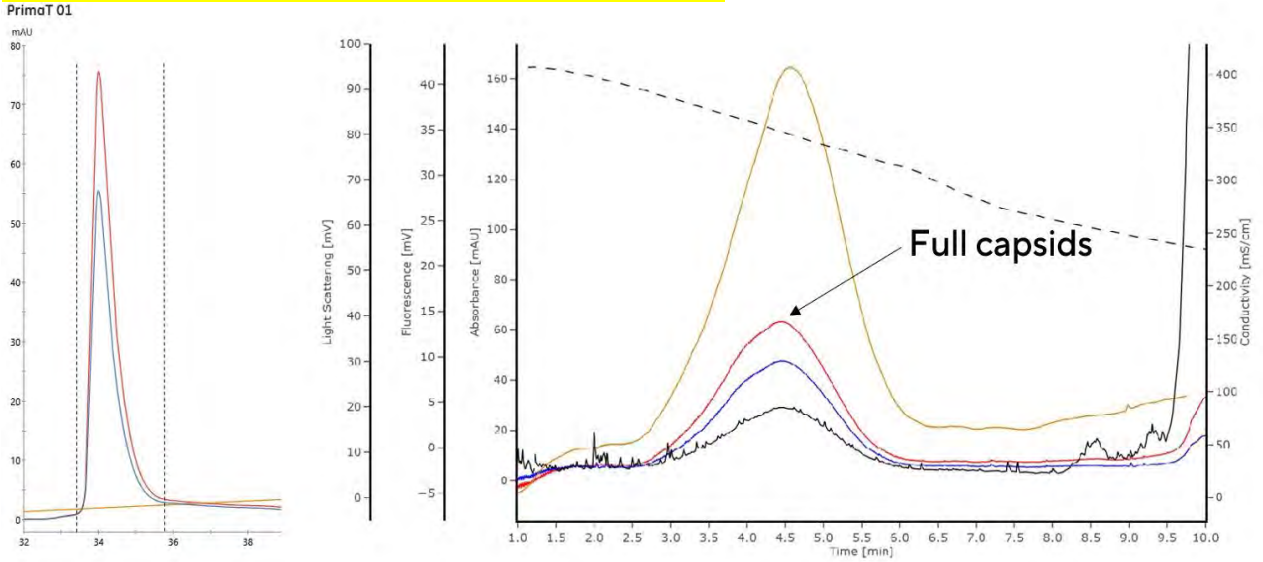
SO3 elution fraction loaded on ultracentrifuge

LC chromatogram **Centrifugram**



PrimaT elution fraction loaded on ultracentrifuge

LC chromatogram **Centrifugram**



Ultracentrifuge analysis in CsCl gradient of AAV with concentration 5E+11 vg (determined by ddPCR). Method: spin for 24h at 50.000g. **Centrifugram using PATfix detectors** (red line – 260 nm absorbance, blue line – 280 nm absorbance) in conjunction with fluorescence (orange line – intrinsic tryptophan fluorescence) and MALS detector (black line – MALS signal) was used for visualisation of separated fractions.

Recovery of the PrimaT column: 100% by ddPCR

Conclusions

- Advance **removal of chromatin dramatically improves performance and reproducibility of all chromatography methods**, at capture and continuing into separation of empty and full capsids.
- Pre-staining analytical samples with **Picogreen enables SEC and ion exchange methods to provide easy and sensitive monitoring of DNA content** across purification processes.
- **Ultracentrifugation and chromatography support orthogonal analysis**. Combined with multiple detectors, both provide deeper insight into sample composition.
- **PrimaT offers new options for removal of empty and damaged capsids,** and also contribute to better clearance of contaminating DNA and endotoxins.

Thank you for your attention!

Please visit our virtual booth

PDF presentation available from:

https://www.biaseparations.com/en/library/seminars-webinars/1119/chromatin-dna-removal-from-harvest-before-an-aav-capture-step-greatly-improves-robustness-purity-and-yield-of-the-overall-downstream-process

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BIA separations

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