BIA separations

Celebrating 20 Years of Innovation

Enabling Tools for Purification and Understanding Complex Biomolecules Structure

Ales Strancar

BIA Separations has been funded to serve the needs of gene therapy and during 2018 is celebrating 20 years of innovation.

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About BIA Separations

- Incorporated in September 1998 in Ljubljana, Slovenia.
- Formed an OEM partnership with Agilent in 2008.
- Moved to dedicated new facility in Ajdovščina in 2011.
- Became the first biopharmaceutical drugs manufacturing for marketing supply using CIM monoliths in 2016/2017.
- End of 2017 Pete Gagnon, lead purification expert, editorial advisor to BPI and GEN, moved to Ajdovščina and took the position of the new CSO.
- On track to become a *single source provider* of expertise for gene therapy, vaccines, VLPs, phages, exosome, RNA, DNA,...

BIA Separations location

BIA resides in the foothills of the Alps across the border from Austria, about 100 miles East from Venice.

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BIA Separations products and services

Convective Interaction Media (CIM®)
Pre-packed monolithic columns
CIMac™ Analytical and CIMmultus™ Preparative columns

Services, Process development and Technical Support
Development of processes and methods for separation/concentration/purification of large biomolecules.
Custom immobilization, product development,..

Process Analytical Technology (PATfix™)
At-line PAT HPLC suite for **faster process development** and enhanced process control

Integrated Capability from Cell Culture Production through Downstream Processing
Bioprocess scale-up from laboratory to pilot
Managing interface between upstream and downstream
Vero cell bank

Bioprocess knowledge

- pDNA (plasmids larger than 30 kbp)
- mcDNA (shorten the pDNA)
- ssRNA and dsRNA
- Adeno virus
- AAV (all serotypes)
- Influenza virus (all serotypes)
- Vaccinia/MVA
- Exosome
- Bacteriophage
- IVIG
- IgM and many more

BIA Separations State-of-the-Art Production Facility > 30M USD investment





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Expansion to increase production capacity by 5 till mid 2020 and by 30 till end of 2023 in progress.

Certifications & Approvals

- DMF for DEAE, QA, SO3 , C4 HLD, OH CIM® monoliths and CIMmultus housing on file, others pending
- Partners audits: Baxter-Shire, Novartis-Sandoz, Novartis-AveXis, Octapharma, Boehringer Ingelheim, Teva, Agilent, and many at present still confidential partners.
- FDA audited to meet to USA GMP regulations
- ISO 9001: 2008

Convective Interaction Media (CIM) monolithic columns

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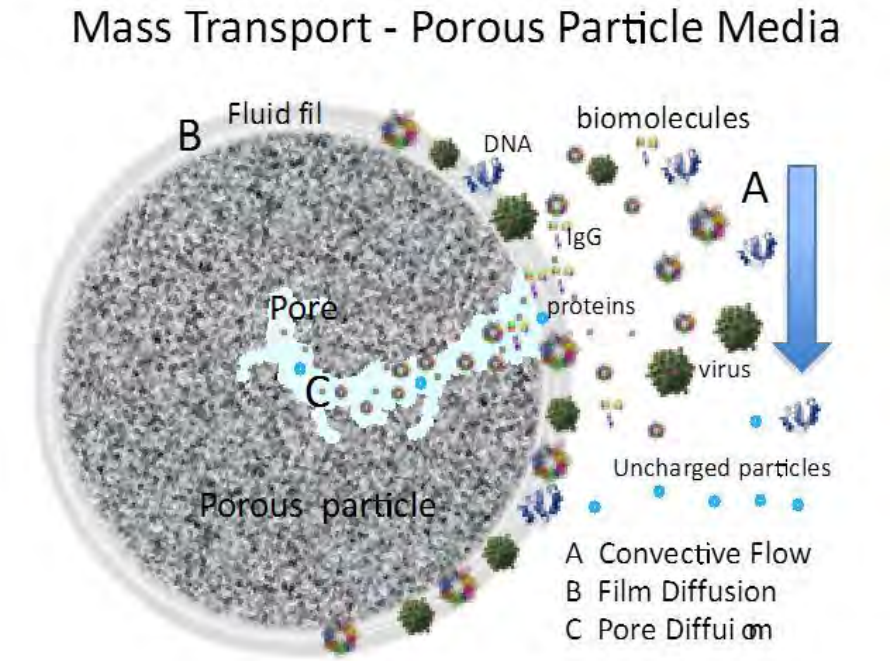
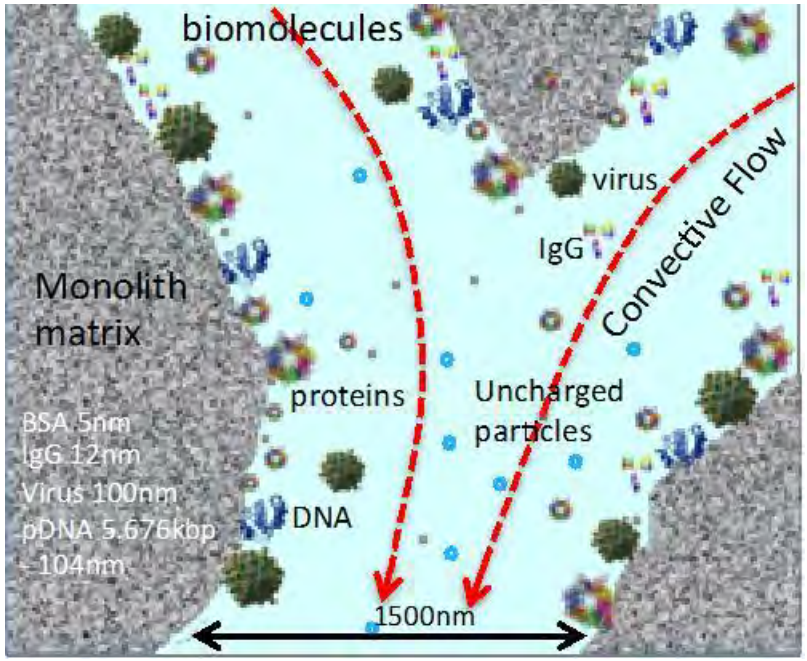
Instant chromatography and enzymatic processes using monolithic resins – no diffusion constraints





Traditional approach - Porous particle:

1. Diffusive mass transport – slow process or lower resolution
2. Pores too small – very low capacity
3. Counter current flow - shear forces – lower yields

Novel UNIQUE approach – Monolithic columns:

1. Convective mass transport – flow independent resolution and capacity, very fast processes
2. Accessible surface for big molecules – high capacity
3. Laminar flow - No shear forces – better yields of e.g. IgM, Lenti, Adeno, Vaccinia, Flu,...
4. And better resolution due to lack of diffusion and no turbulent mixing

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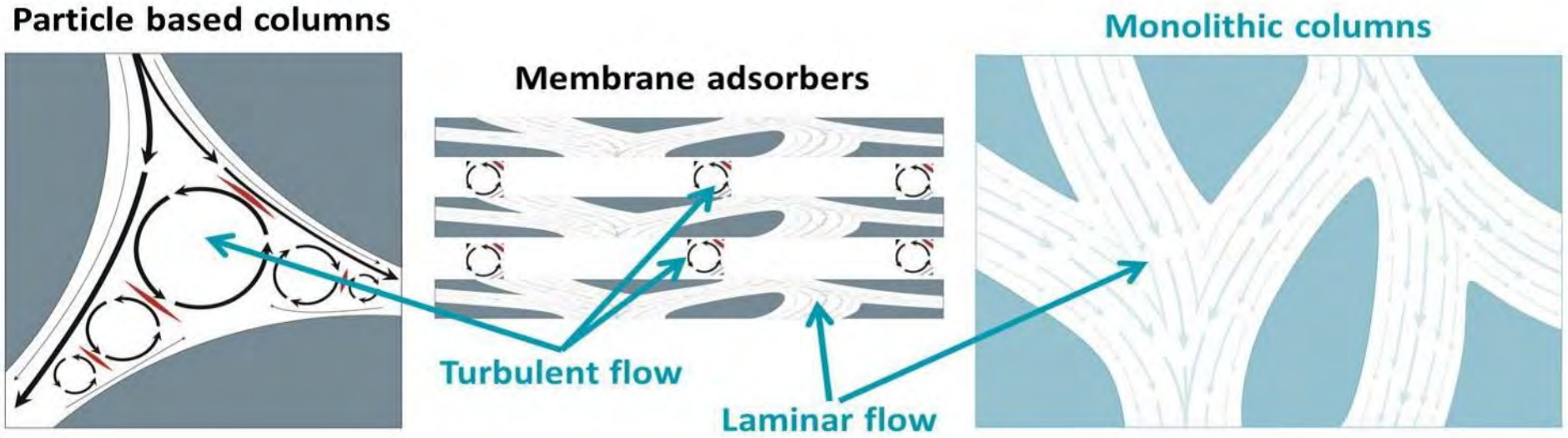
Shear stress is very low in non-porous monoliths



Particle columns and membranes are dominated by turbulent shear stress

Turbulent shear stress in porous particle columns and membranes is directly proportional to flow rate and mobile phase viscosity. Both increase the grinding intensity at countercurrent interfaces and **destroy shear sensitive products.**

Flow through monoliths is almost exclusively laminar. Lack of turbulent shear contributes to higher recovery of infective / potent virus and better purity due to low back mixing.

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Lower shear stress in monoliths results in higher live influenza A virus recovery

Average values	QA monolith	Q membrane	Q porous particles	semi-affinity porous particles
Virus Recovery	54%	35%	35%	27%
DNA Depletion	96%	95%	95%	91%
Protein Depletion	95%	94%	98%	99%
Dynamic Binding Capacity	**10.3** $\log_{10}$ TCID50/mL Support	**10.3** $\log_{10}$ TCID50/mL Support	**9.0** $\log_{10}$ TCID50/mL Support	**8.4** $\log_{10}$ TCID50/mL Support

50% better recovery results in e.g. 1,5 M doses of vaccine instead of 1 M doses, at the same costs of the process = 0,5 M doses are pure profit

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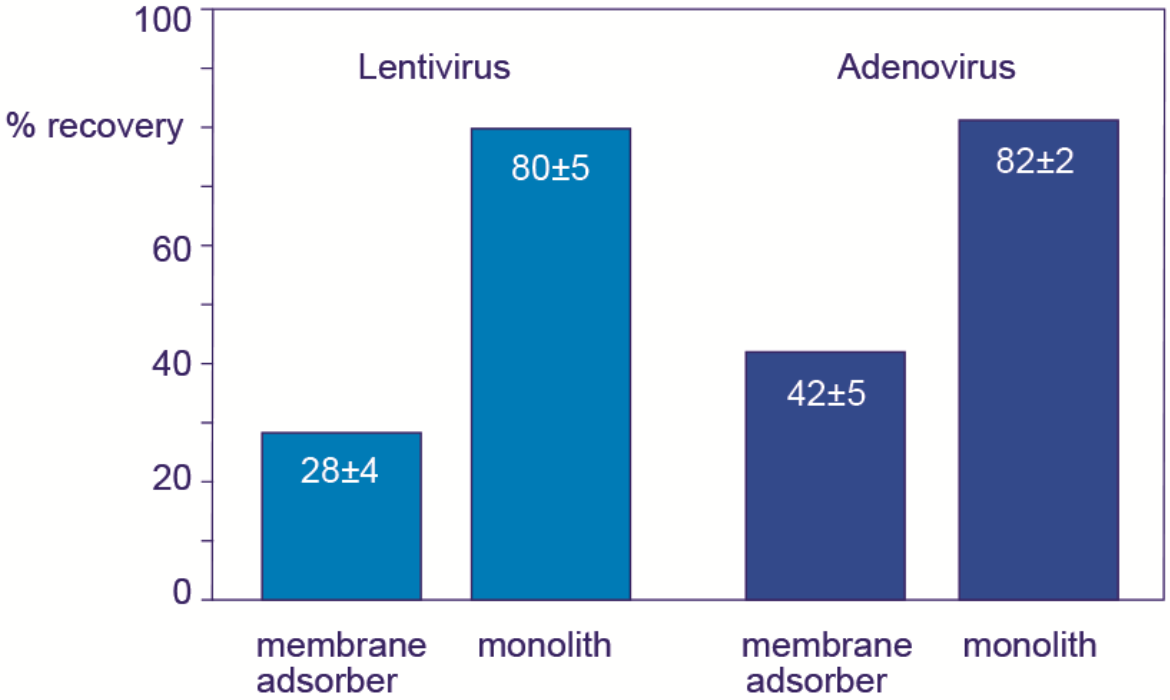
Maurer et al., Purification of Biological Products, Waltham, MA/USA, 2007

Lower shear stress in monoliths results in higher virus recovery compared to membranes

Comparison of anion exchange monoliths and membrane adsorbers



Bandiera et al, Downstream processing of lentiviral vectors: releasing bottlenecks, Hum. Gene Ther. Met. 23 (2102) 255–263.

Fernandez et al, Bioprocessing development for canine adenovirus type 2 vectors, Gene Ther. 20 (2013) 353–360.

High ligand density, no diffusion, no turbulent mixing - separation of empty and full capsids

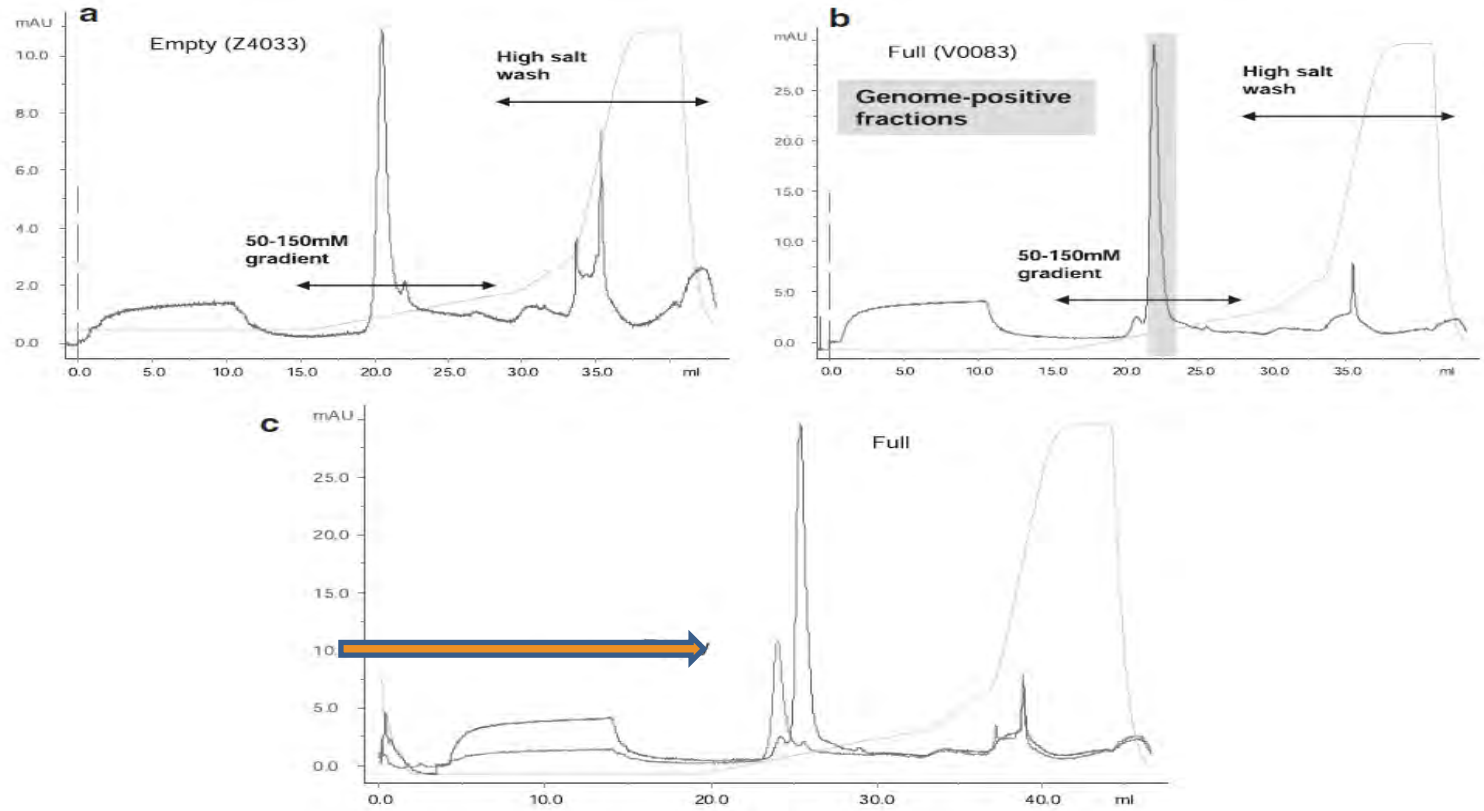
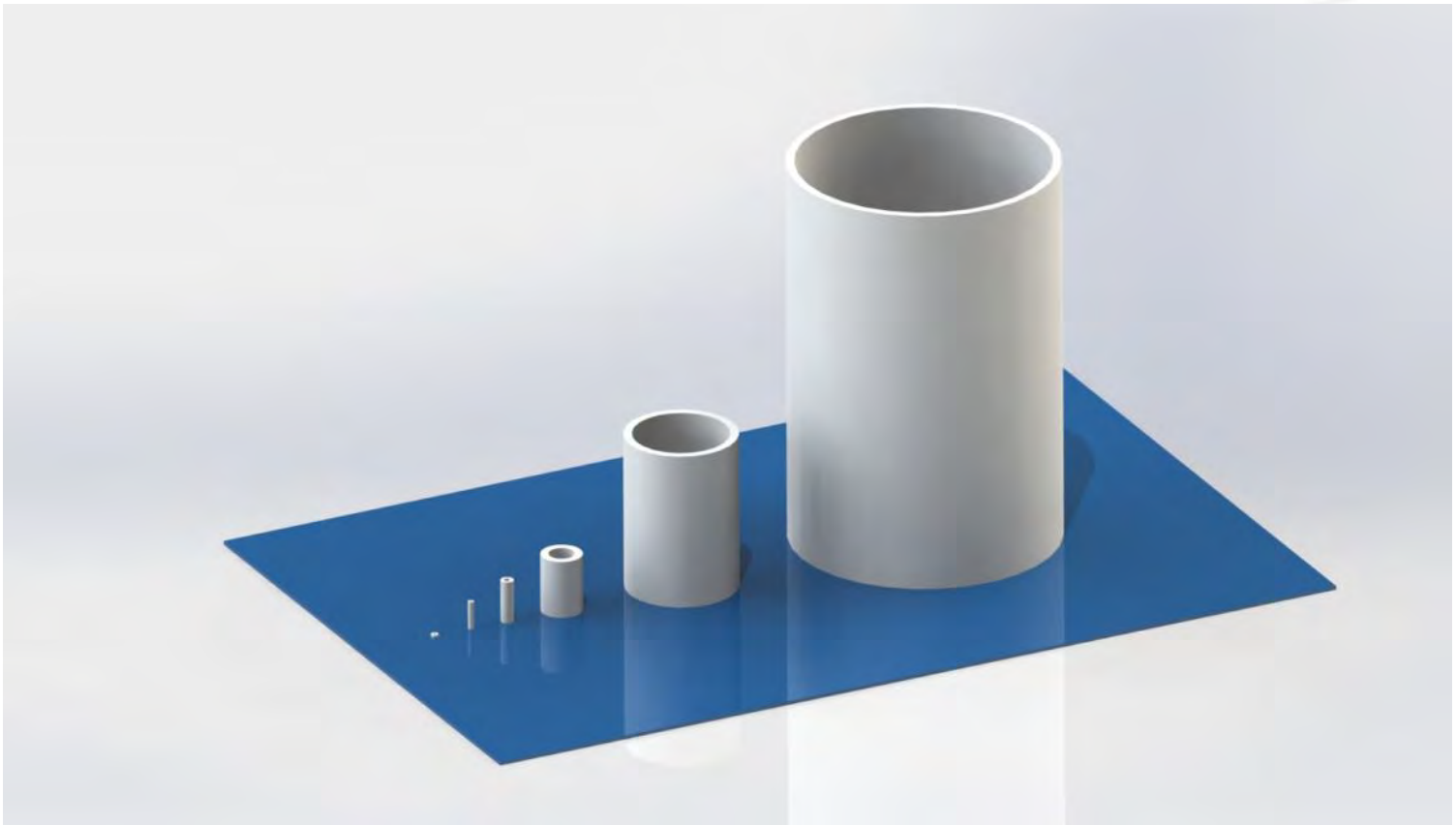


FIG. 3. IEX particle assay. **(a)** Seventy-five microliters of an empty particle AAV8 preparation (lot no. Z4033) was loaded onto a 0.34-ml CIM-QA disk, using FPLC, and eluted with a 50–150 m*M* salt gradient. The y axis shows the absorbance (mAU) at 280 nm and the *x* axis the elution volume (ml). The detected conductivity and absorbance are represented by solid light and dark blue lines, respectively. The vertical dashed pink line represents the point of vector injection. **(b)** A full AAV8 vector preparation (lot no. V0083, 1×10^{12} GC) was run under the same binding/elution conditions as used for the empty particle preparation. Fractions were quantified for vector GC content and those fractions containing >99% of the loaded material are indicated (shaded box). **(c)** An overlay of the elution profiles of the empty and full AAV8 vector preparations is shown.

Lock et al., HUMAN GENE THERAPY METHODS: Part B 23:56–64 (February 2012)

CIM® monoliths are (easily) scalable, up to 40L, at present



	1 mL	8 mL	80 mL	800 mL	8 L	40 L
I.D. (mm)	6.4	6.5	16.2	65	243	636
O.D. (mm)	18.3	14.4	33	105	285	680
Thickness (mm)	5.95	3.95	8.4	20	21	22

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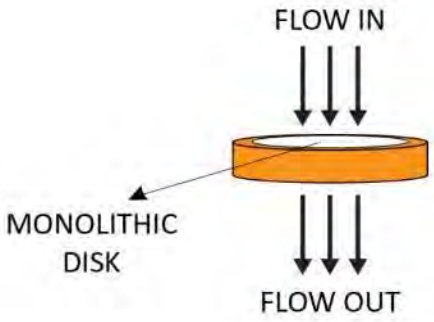
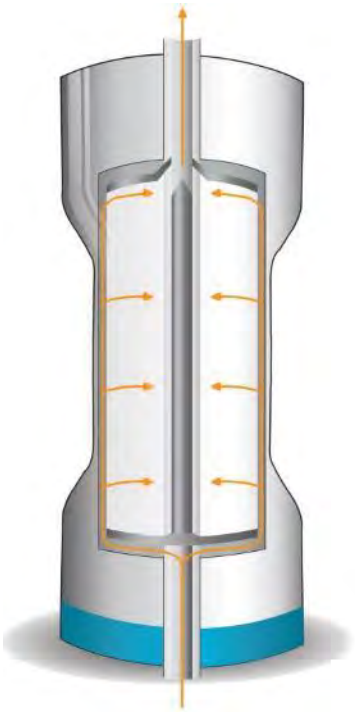
Flow geometries

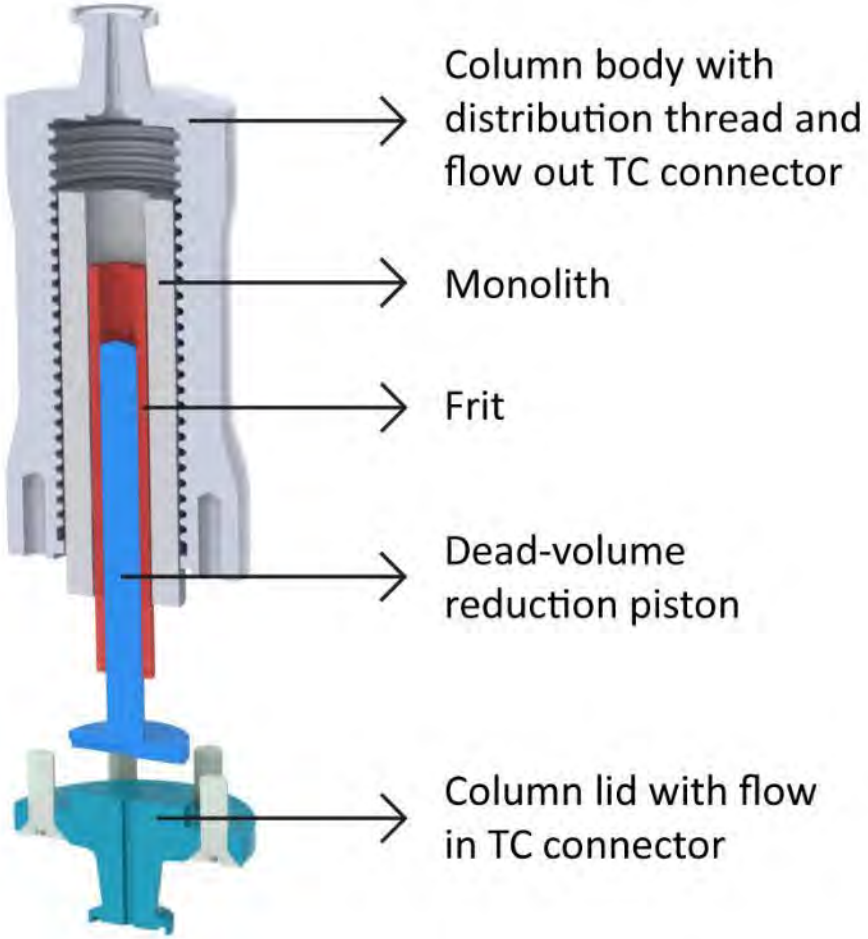
Axial flow



Radial flow



CIM® Tube Monolithic column structure



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Introduction of composite materials to combine advantages of SS and plastics

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

- **Disposable but multiuse**
- **Stainless steel performance characteristics**
- **cGMP compliant**

allows for robust continuous operations

CIMmultus™ composite materials – matching stainless steel characteristics

	1 mL		8 mL		80 mL		800 mL		8000 mL	
Type of column	CIM® SS	CIMmultus™	CIM® SS	CIMmultus™	CIM® SS	CIMmultus™	CIM® SS	CIMmultus™	CIM® SS	CIMmultus™
Max pressure	18 bar	18 bar	20 bar	20 bar	20 bar	20 bar	14 bar	14 bar	7 bar	7 bar
Recommended flow rates (mL/min)	5	5	50	50	220	220	1100	1100		
Max flow rate (mL/min)	16	16	100	100	400	400	2000	2000	8000	8000
Max operating temperature	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C	40 °C
Storage conditions	Chemistry specific, consult Product Sheet. 20 % ethanol for IEX.									
Sanitisation	Chemistry specific, consult Product Sheet. 1 M NaOH for at least two hours for IEX, C4 HLD.									

BUT:

- **3 times cheaper**
- **5 times lighter**
- **allow for pre-packed column transport**
- **customer decides to use disposable column as single or multi use unit**

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CIM® Monolithic columns

CIMmultus (multi-use disposable columns)

0.1 | 0.34 | 1 | 8 | 80 | 800 | 8000 ml

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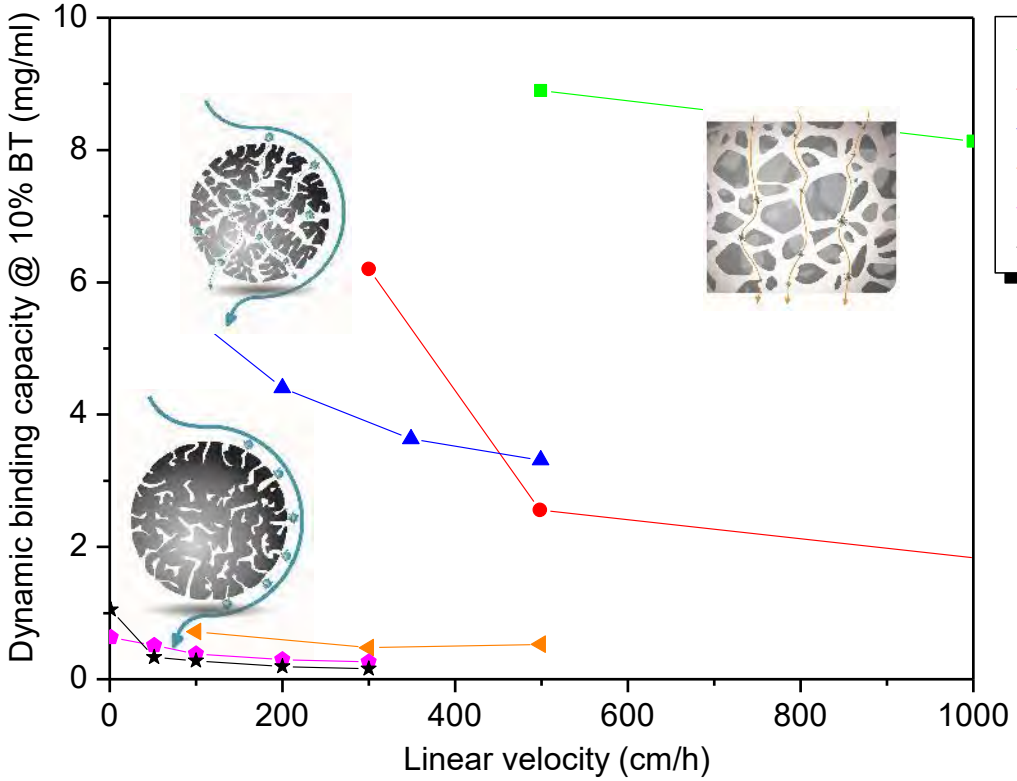
autoclavable

Boehringer Ingelheim reports 15-fold increase in productivity using CIM® DEAE columns



CIM® DEAE binding capacity
= ~8 mg/ml

Used for CP III trials

Boehringer Ingelheim: „15-fold increase in productivity“

- High binding capacity at relevant flow rates
- High elution concentration - pDNA eluted in lower volume (important for SEC!)
- Fast process (no product loss due to oxidative degradation or enzymatic attack)

Urthaler et al., J.Chrom. A, 1065 (2005), 93-106

Economic benefit for the customer using CIM® Monolith plasmid DNA purification pack

CIM monolith – BIA Separations

Calculations	
Buffer	76,3 ml buffer/mg pDNA
Time	23,6 min /mg pDNA
Recovery	85%
Costs using column for 1 run	
Quantity of purified pDNA	5,10 mg PDNA
€ (Column costs)	114 €/mg pDNA
€ (Column + buffer)	114 €/mg pDNA
Costs using columns for 10 runs	
Quantity of purified pDNA	51 mg pDNA
€ (Column costs)	11,4 €/mg pDNA
€ (Column + buffer)	11,8 €/mg pDNA
Costs using columns for 20 runs	
Quantity of purified pDNA	102 mg pDNA
€ (Column costs)	5,7 €/mg pDNA
€ (Column + buffer)	6,1 €/mg pDNA
€ (column + buffer+ work)	15,4 €/mg pDNA

CIM® monolithic columns offer **3 times cheaper** purification costs of pDNA for gene therapy

Particle based

Calculations	
Buffer	108,0 ml buffer/mg pDNA
Time	70,0 min /mg pDNA
Recovery	79%
Costs using column for 1 run	
Quantity of purified pDNA	4 mg PDNA
€ (Column costs)	227 €/mg pDNA
€ (Column + buffer)	228 €/mg pDNA
Costs using columns for 10 runs	
Quantity of purified pDNA	40 mg pDNA
€ (Column costs)	23 €/mg pDNA
€ (Column + buffer)	24 €/mg pDNA
Costs using columns for 20 runs	
Quantity of purified pDNA	79 mg pDNA
€ (Column costs)	11 €/mg pDNA
€ (Column + buffer)	12 €/mg pDNA
€ (column + buffer+ work)	42 €/mg pDNA

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PATfix™

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

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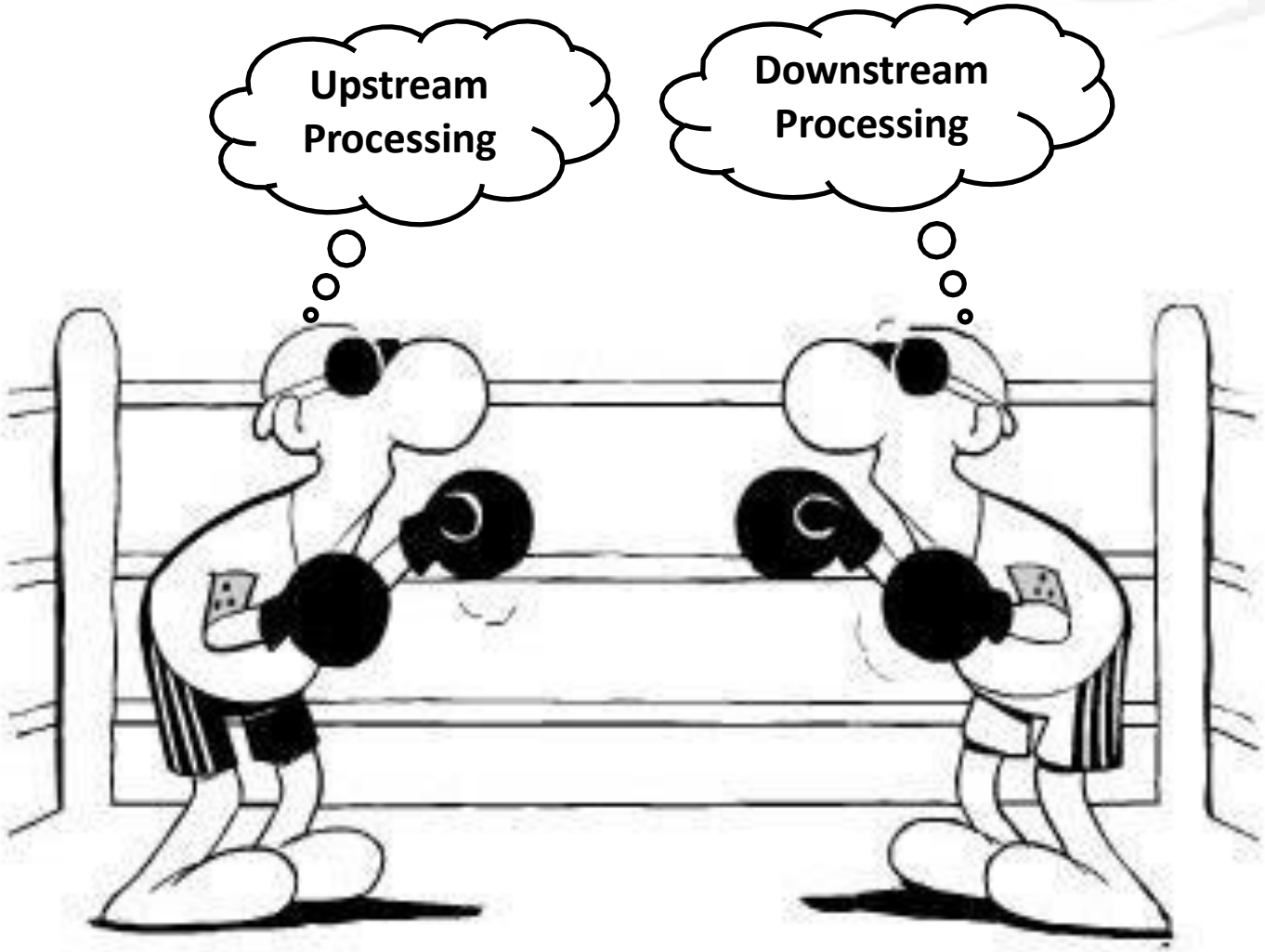
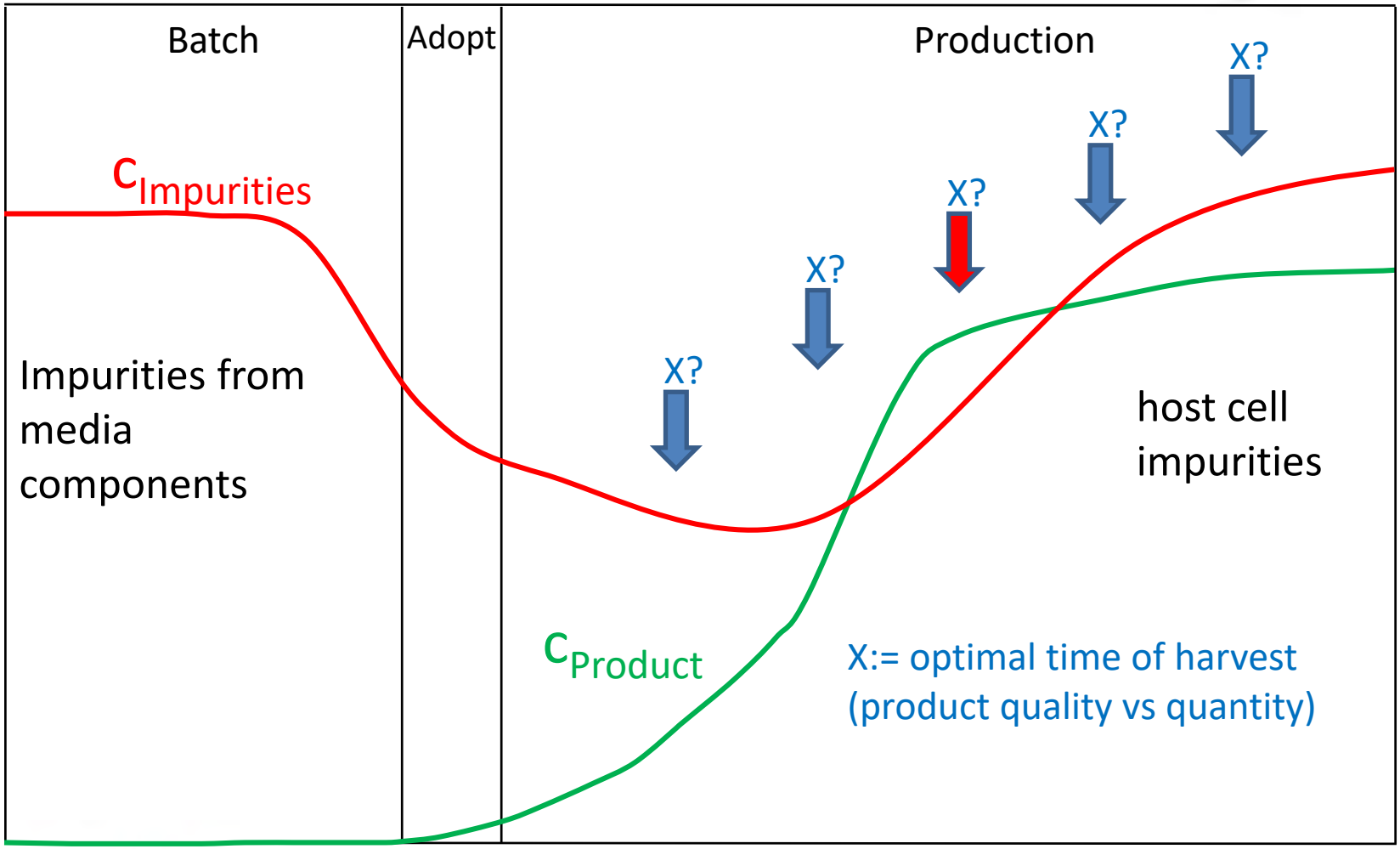
Reality in bioprocessing



PAT HPLC – ultimate goal to identify optimal harvesting point - the DSP should master the USP!



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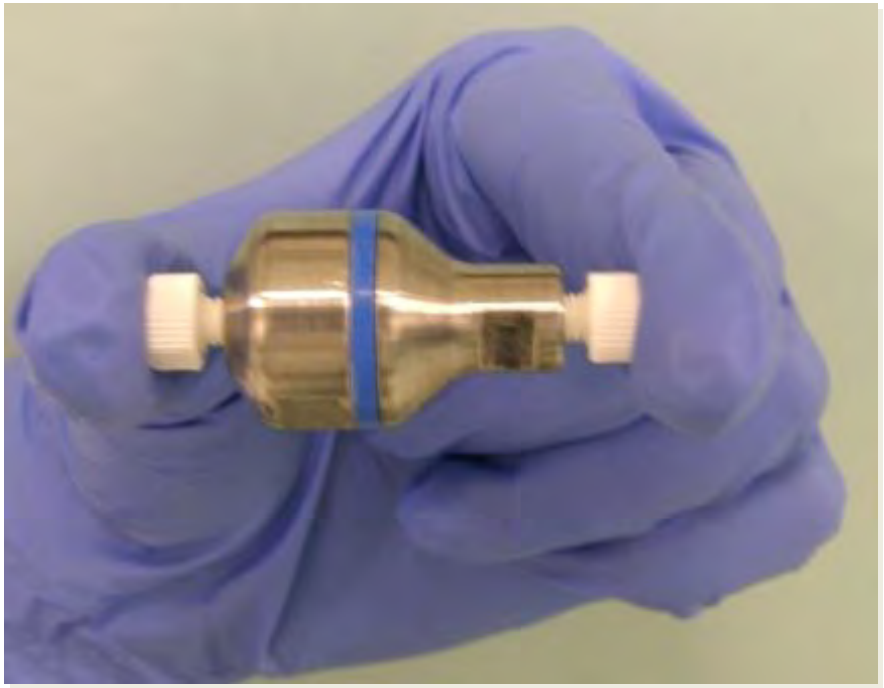
Proper knowledge and thorough understanding of the process is prerequisite for a robust process;

accurate and fast analytic is a fundament to accomplish.

Use of the HPLC for accurate process mass balance calculation – *mandatory for robust process*

CIMac™ / Bio-monolith™ HPLC columns



10 ml/min = 4500 cm/h = 360 CV/min, res. time 0.1 s*): faster than biosensor*

No entrapment in the column: *no sample carry-over*

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CIMac™ analytical columns for PAT HPLC

no carry over of contaminants or viral particles

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

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BIA Separations PATfix™

Dedicated HPLC

CIMac™

inCyght

for chromatography

Integrated monitoring to detect changes and quantify complex analytes.

Custom tailored system to meet requirements of bioanalytical HPLC techniques.

Allows for different detectors integration (UV, pH, conductivity, Fluorescence, MALS,...)

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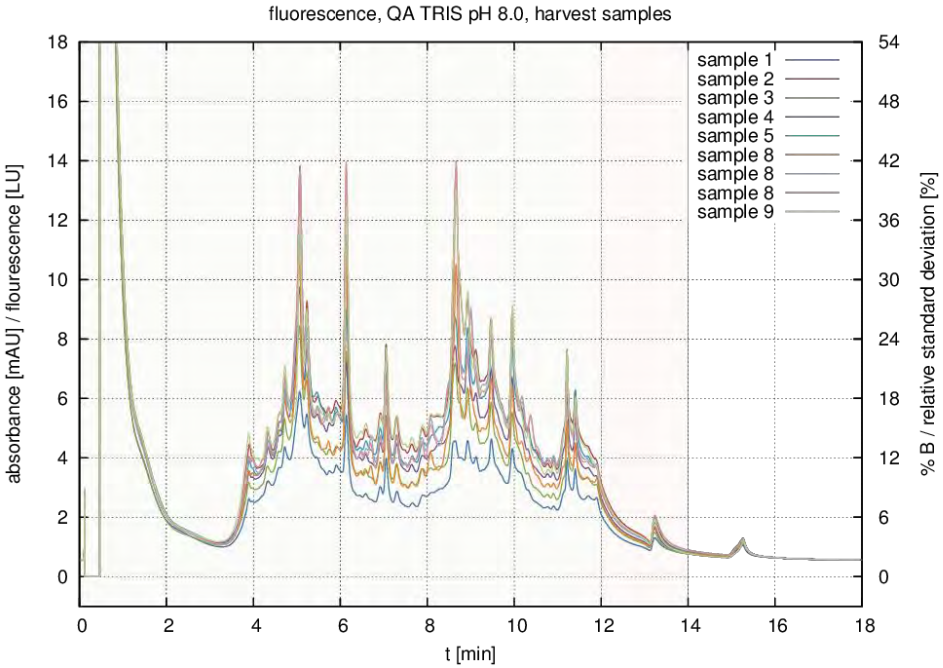
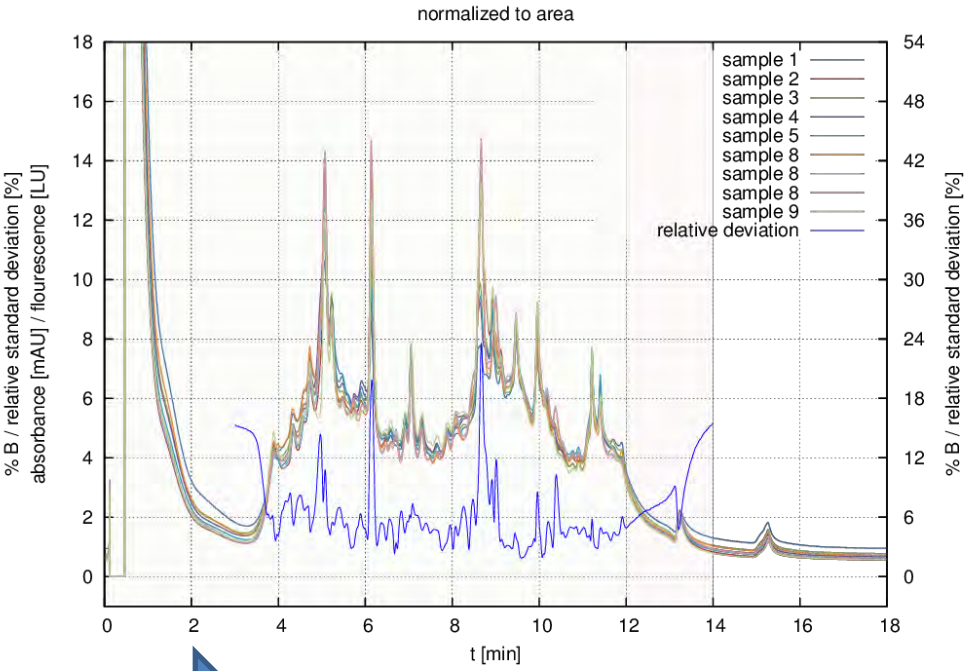
PATfix™ Fingerprint approach to study robustness of the AAV fermentation scale-up, *from 5L to 100L*





Normalized chromatograms

Average relative standard deviation of all area normalized fingerprints is 5.6 %
(including the sample obtained with fermentation at different scales).
One can conclude the fermentation is very robust.

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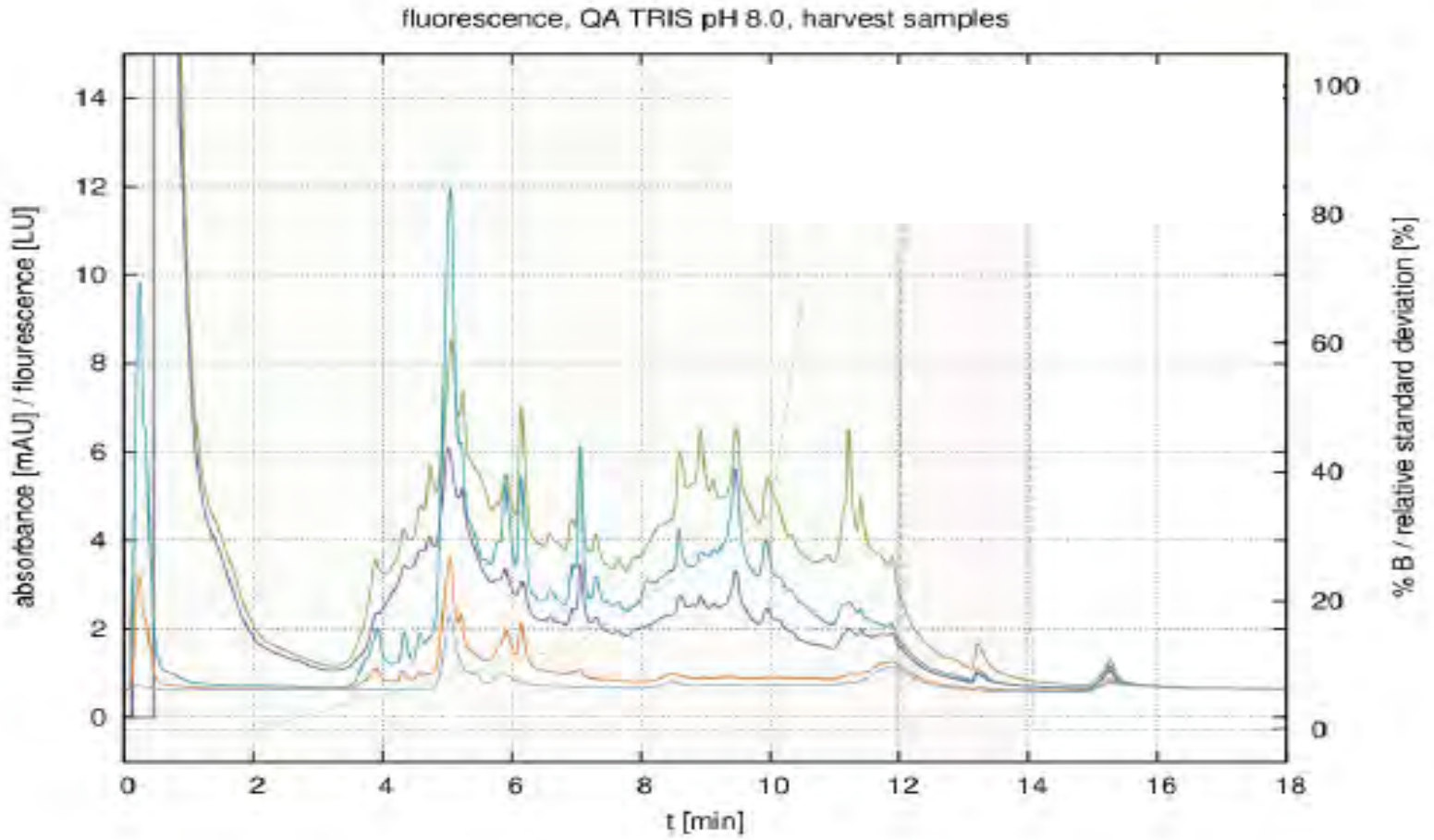
AAV production steps monitoring using fingerprint approach – *secure robustness of each unit operation*



Improved process understanding and control

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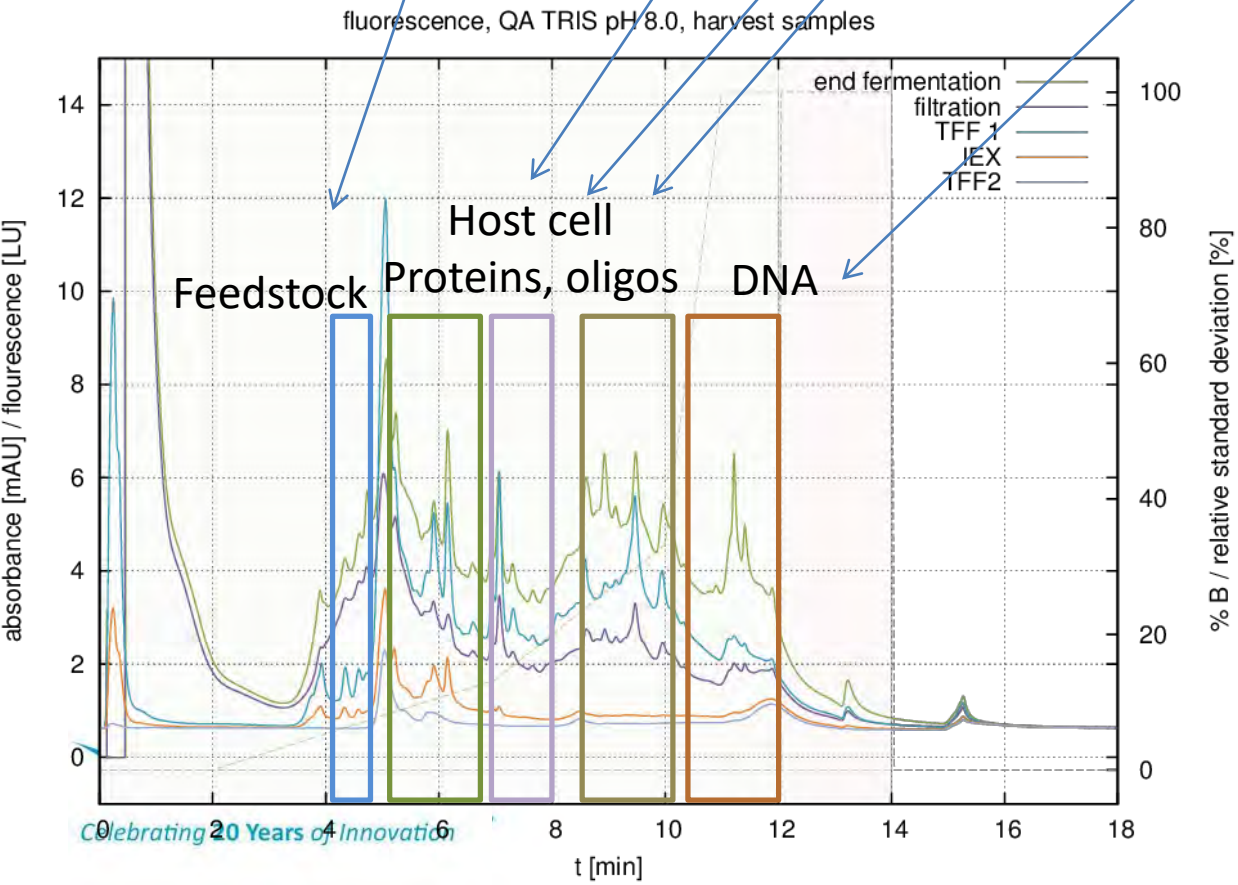
PATfix™ much FASTER and cheaper info than by using traditional methods

PATfix™ fingerprint injection = about 20 € (column + buffers + labor), takes about 20 min

(crio)TEM: 125 €, 30 min
AUC: 200 €, 1 h
SDS-PAGE: 20 €, 4 h
Infectivity: < 10 €, 3-7 days

ELISA: 5 € / well, 4 h
SDS-PAGE: 20 €, 4 h

qPCR: 20 € / well, 3-4 h
ddPCR: 30 € /well, 3-4 h
AGE: 20 €, 2 h



Summary:

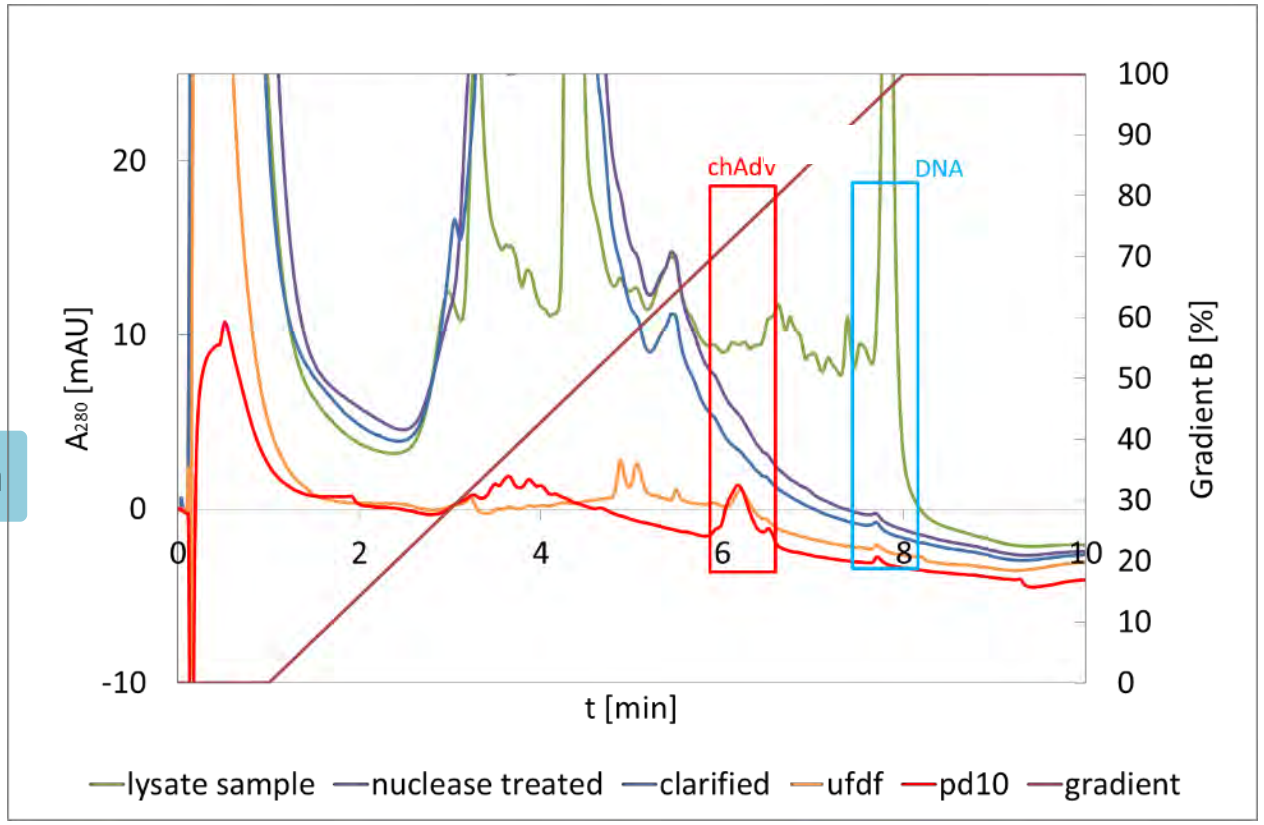
Traditional methods per sample: 100 - 400 €
Time: 1 day to 1 week

PATfix per sample: 20 € per sample
Time: 20 min

No magic but each assay requires optimization.

PAT HPLC - monitor & control of product and impurity profile from up-stream to fill & finish

- Lysis
- Nuclease treatment
- Clarification
- Ultrafiltration/diafiltration
- AEX Chromatography
- Buffer Exchange



- Batch to batch reproducibility
- Product yield and impurity control
- Batch track record

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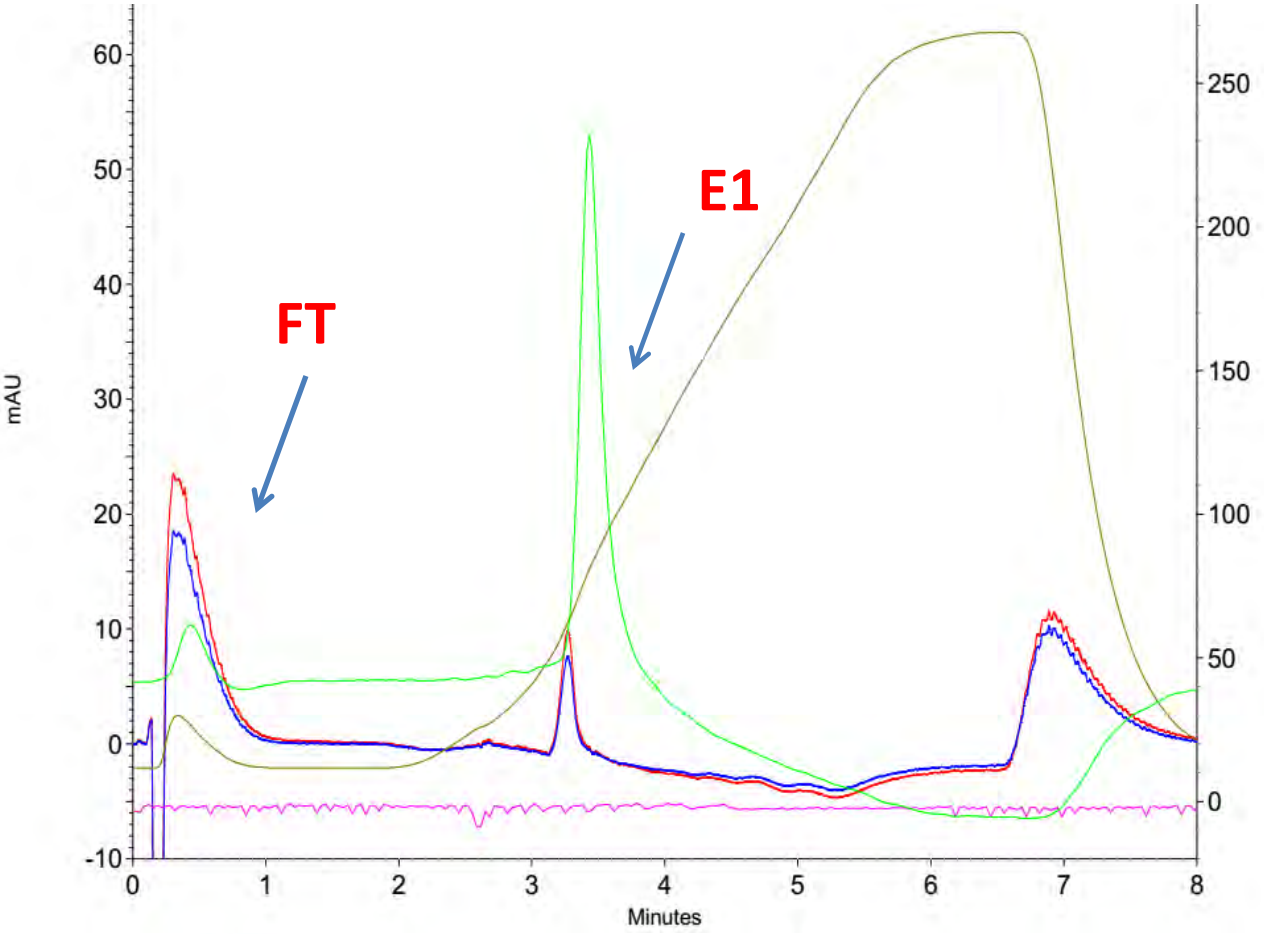
CIMac™ SO_3 - Influenza virus quantity determination – fluorescence detector to improve sensitivity



Influenza A determination
Column: **CIMac™ SO_3**
Flow rate: 1 mL/min
Buffer A: 50 HEPES pH 7.5
Buffer B: 50 HEPES + 1.5 M NaCl, pH 7.5

Curtesy of Blue Sky Vaccines

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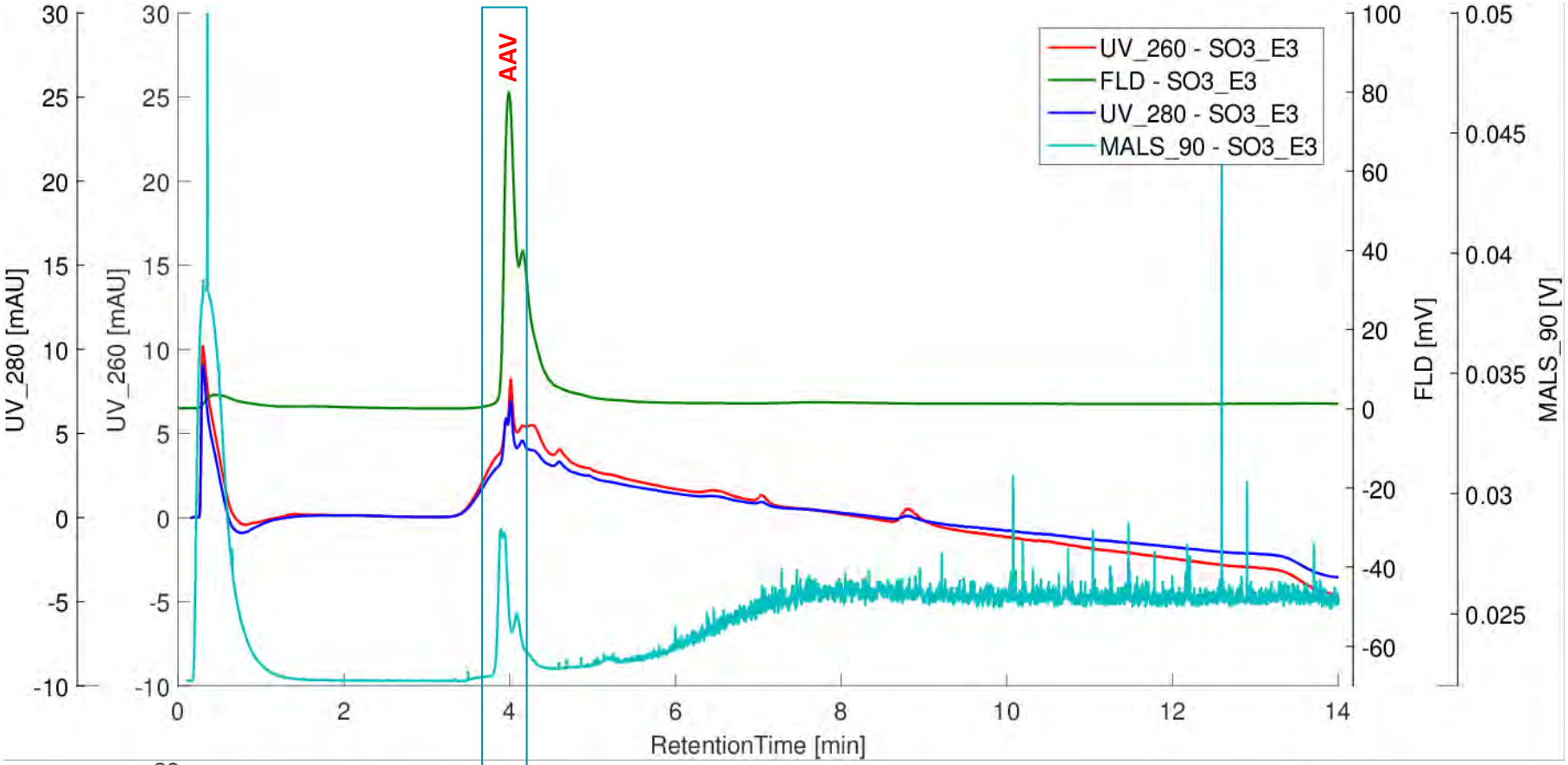
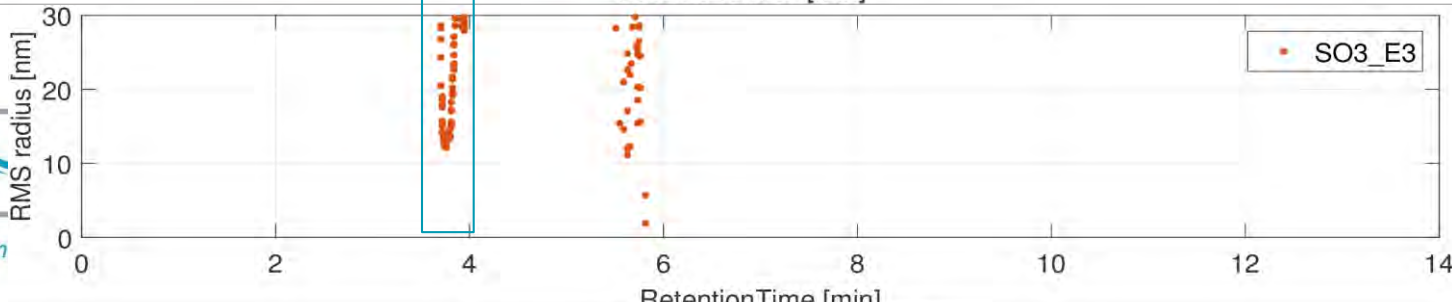
CIMac™ SO_3 - AAV impurity profile – MALS detector to check for aggregates and complexes





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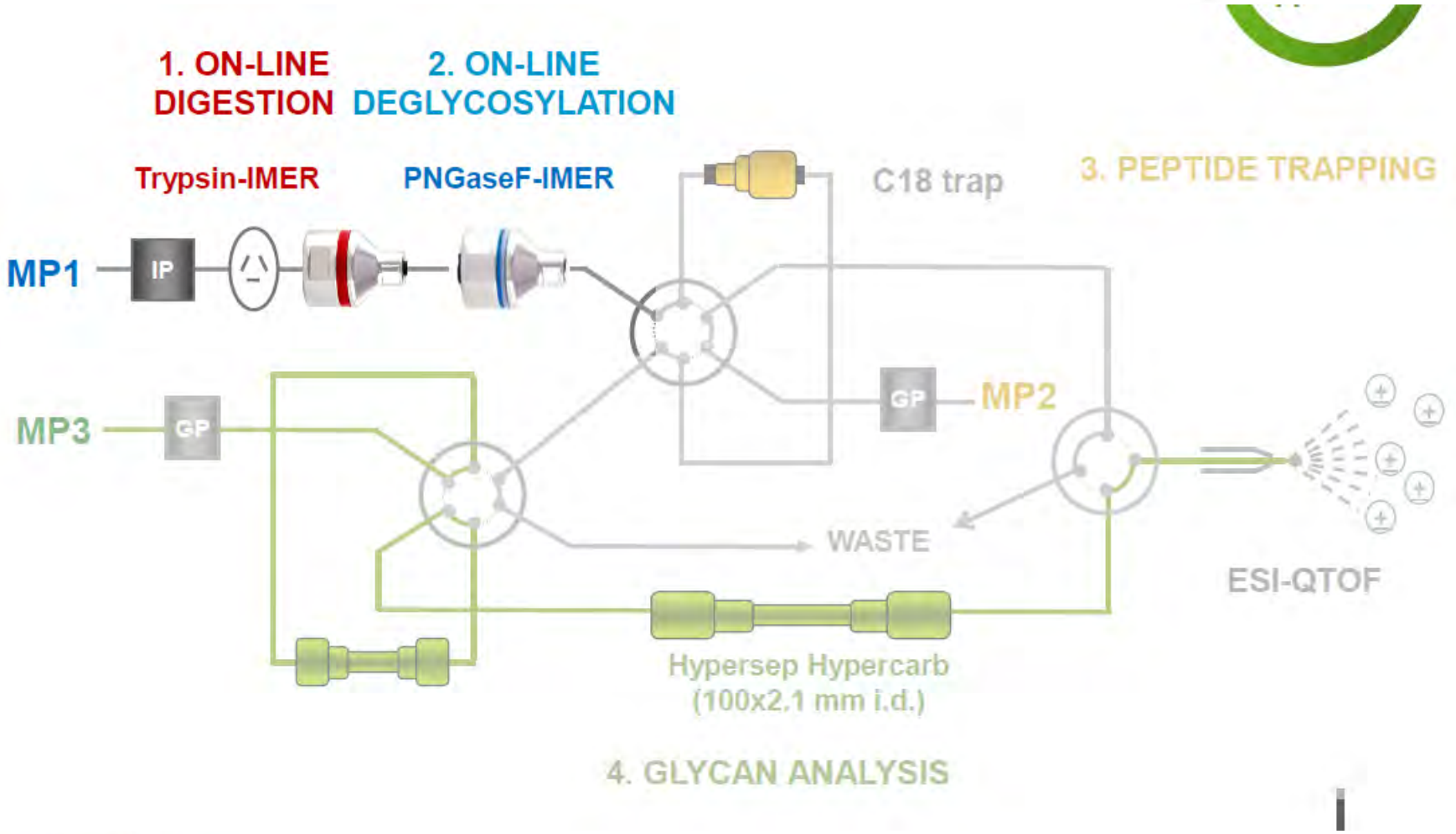
CIM® enzymatic bioreactors – instant bioconversion



Curtesy of Manuela Bartolini, Department of Pharmaceutical Sciences, Via Belmeloro 6, University of Bologna, 40126 Bologna, Italy

2nd generation ccc pDNA Industrial Purification Platform Process

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CIMmultus™ Plasmid Process Pack

E. coli culture with plasmid

↓

Cell harvest

↓

Alkaline lysis with adjustment to
0.5 -1M $CaCl_2$

↓

Clarification

↓

Adjustment to binding conditions

↓

CIM® DEAE

↓

Adjustment with $(NH_4)_2SO_4$

↓

CIM® C4 HLD

↓

Buffer exchange

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1st step: selective RNA precipitation with $CaCl_2$

Alkaline lysis

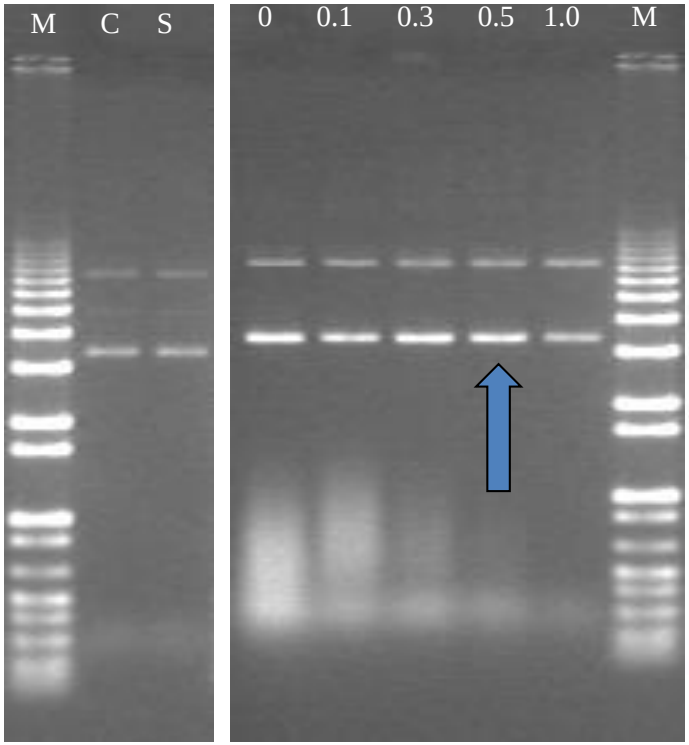
- 50 mM Tris, 10 mM EDTA,pH 8.0
- 0.2 M NaOH, 1% SDS
- 3 M potassium acetate, pH 5.5

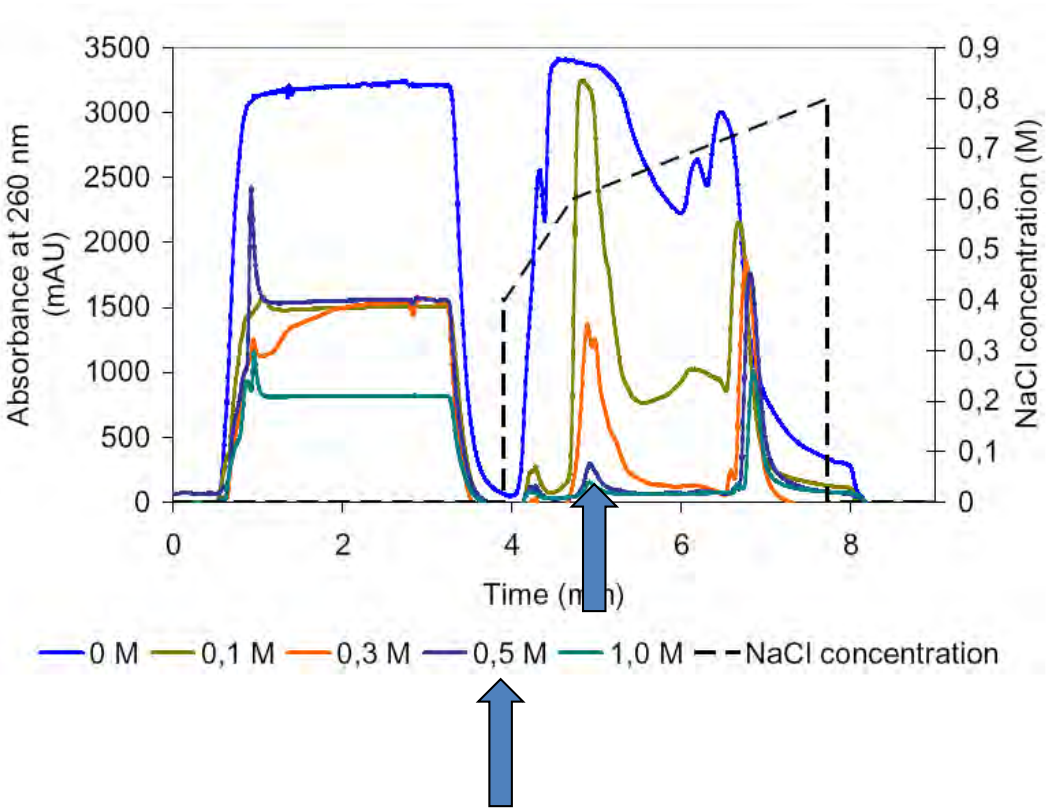
Adjustment (depending on the pDNA size) to 0.5 - 1M $CaCl_2$

Incubation for 15 minutes

Clarification (Centrifugation/Filtration)

1st step: selective RNA precipitation with $CaCl_2$





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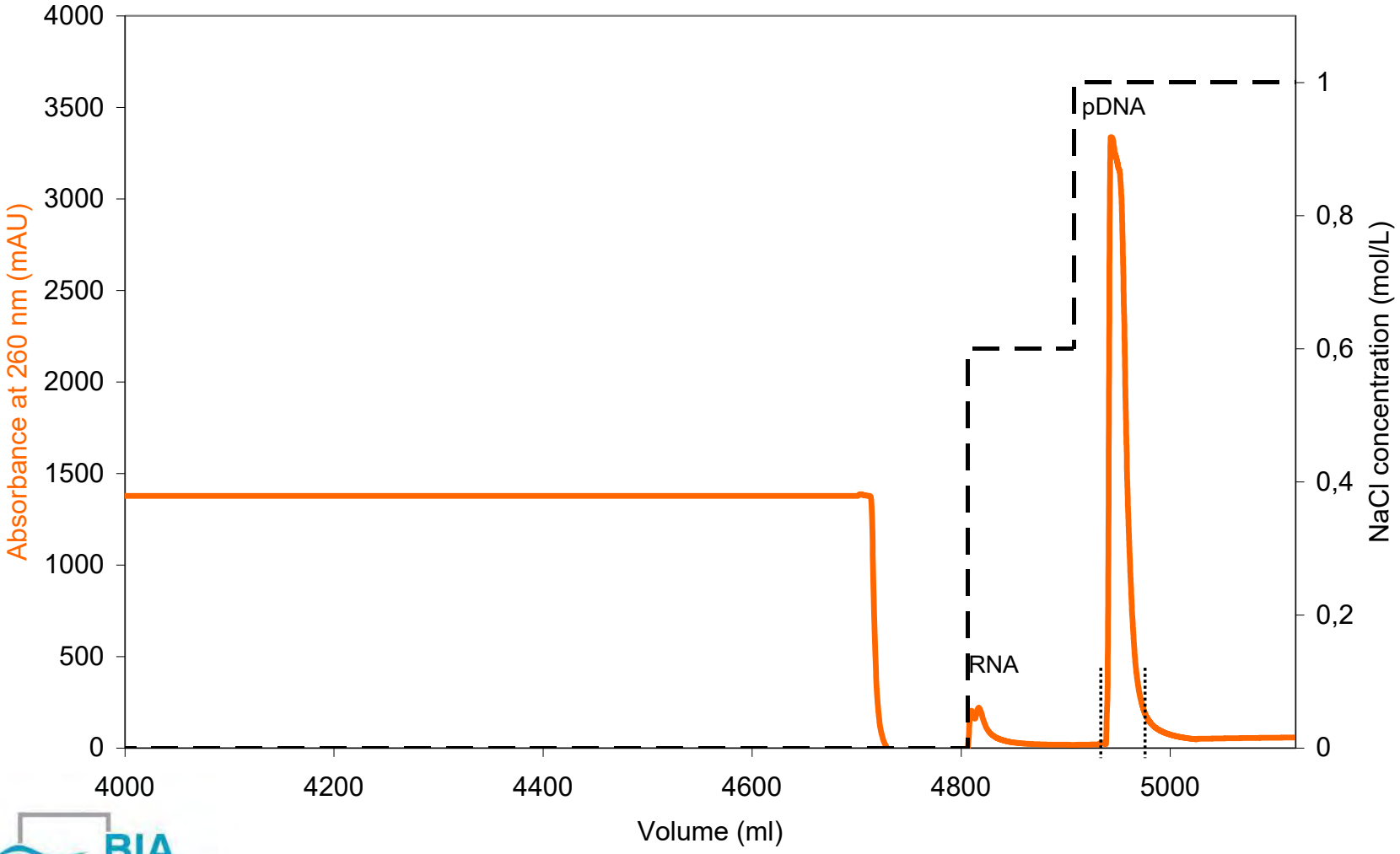
2st step: plasmid DNA capture using CIMmultus™ DEAE column



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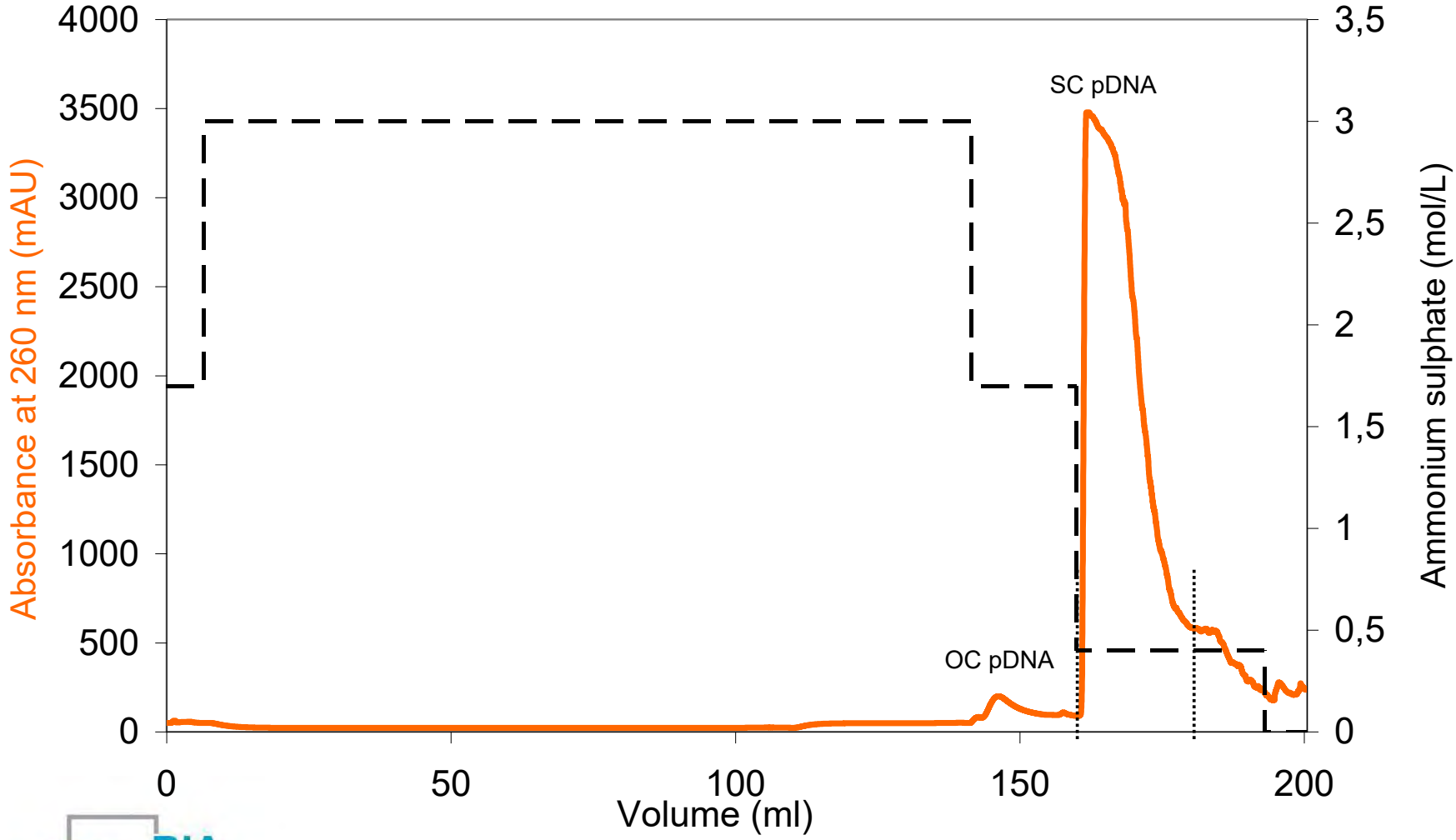
3st step: ccc plasmid DNA isolation using CIMmultus™ C4 HLD column



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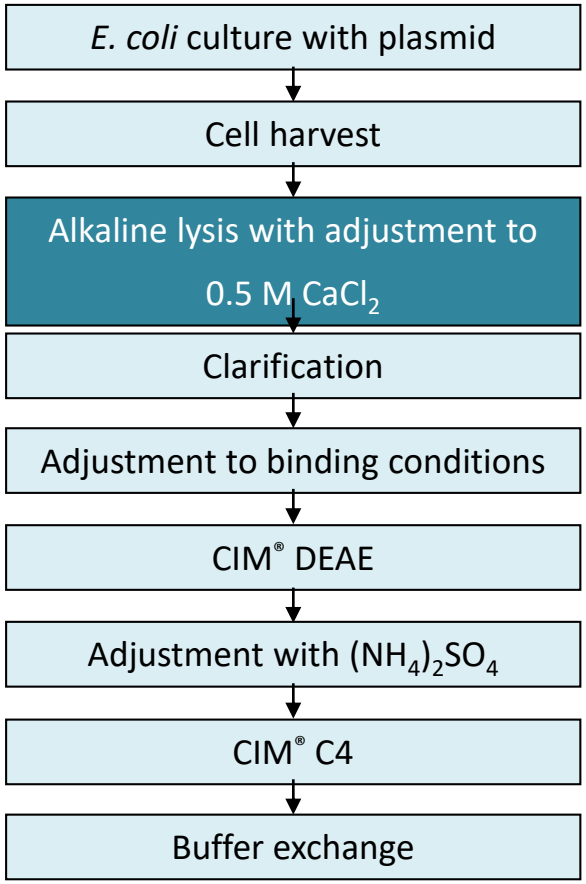
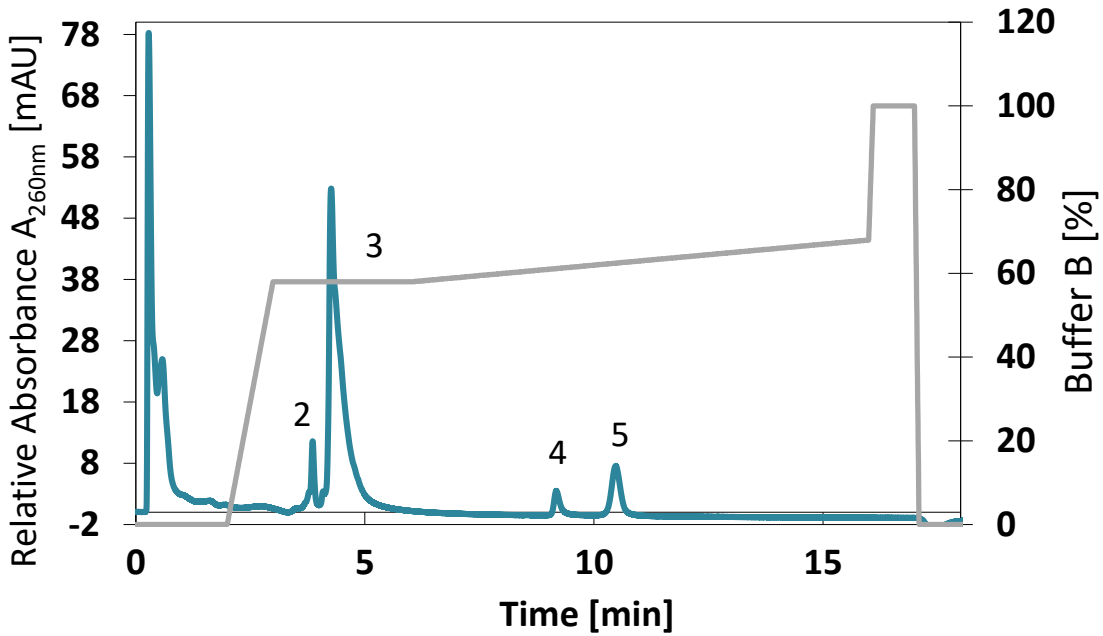
CIMac™ pDNA Analytical Column – alkaline lysis optimisation





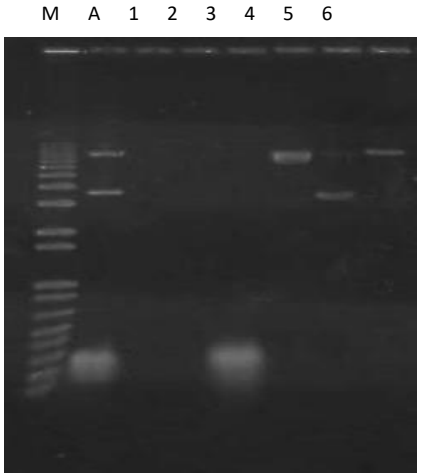


Figure 1: Agarose gel electrophoresis - Molecular weight marker (lane M), sample alkaline lysate plasmid pEGFP-N1 (lane A), peak 1 (lane 1), peak 2 (lane 2), peak 3 (lane 3), peak 4 (lane 4), peak 5 (lane 5), pDNA open circular form standard (lane 6)

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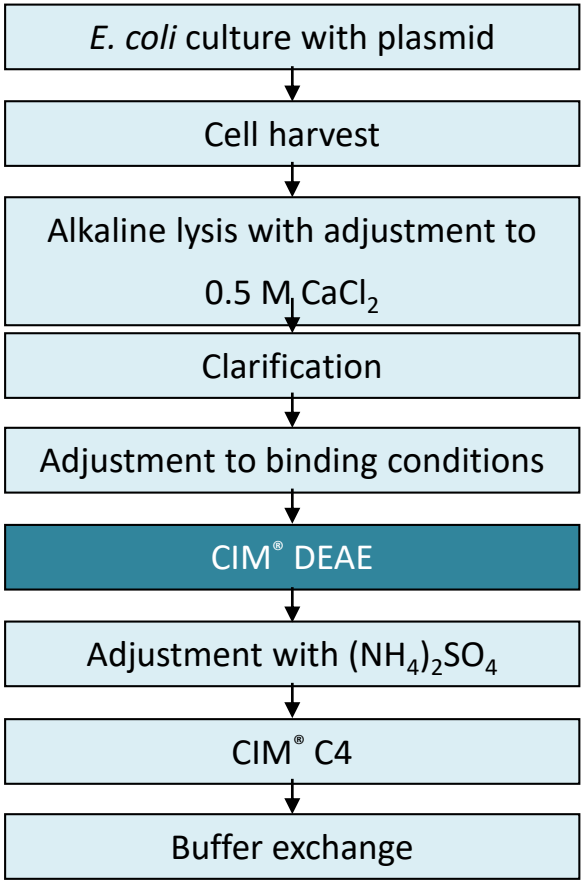
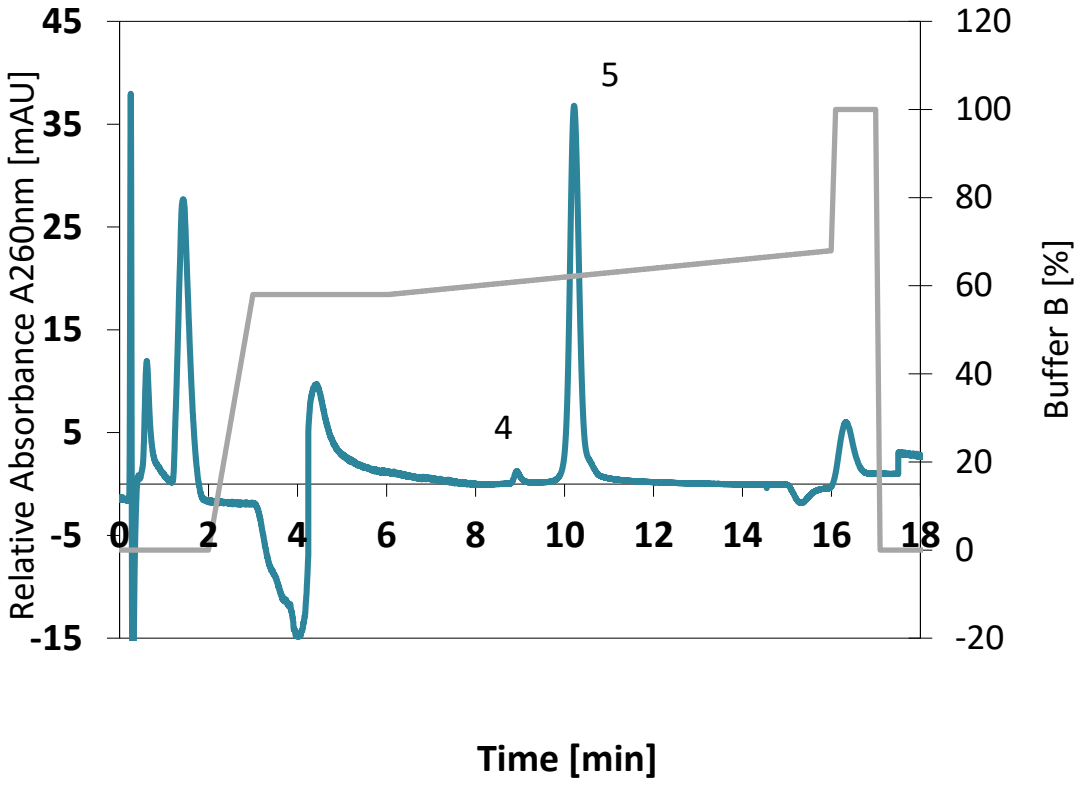
CIMac™ pDNA Analytical Column – 1st chromatography step





Conditions: Flow rate – 1 ml/min; Buffer A – 200 mM Tris pH 8.0 and buffer B – 200 mM TRIS + 1 M NaCl pH 8.0; Injection volume – 20 µl; Sample was diluted 1:3 with water; UV detection – 260 nm; Peak 1 and Peak 2 – other impurities, Peak 3 – RNA, Peak 4 – OC pDNA, Peak 5 – SC pDNA.

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CIMac™ pDNA Analytical Column – 2nd chromatography step

E. coli culture with plasmid

↓

Cell harvest

↓

Alkaline lysis with adjustment to 0.5 M $CaCl_2$

↓

Clarification

↓

Adjustment to binding conditions

↓

CIM® DEAE

↓

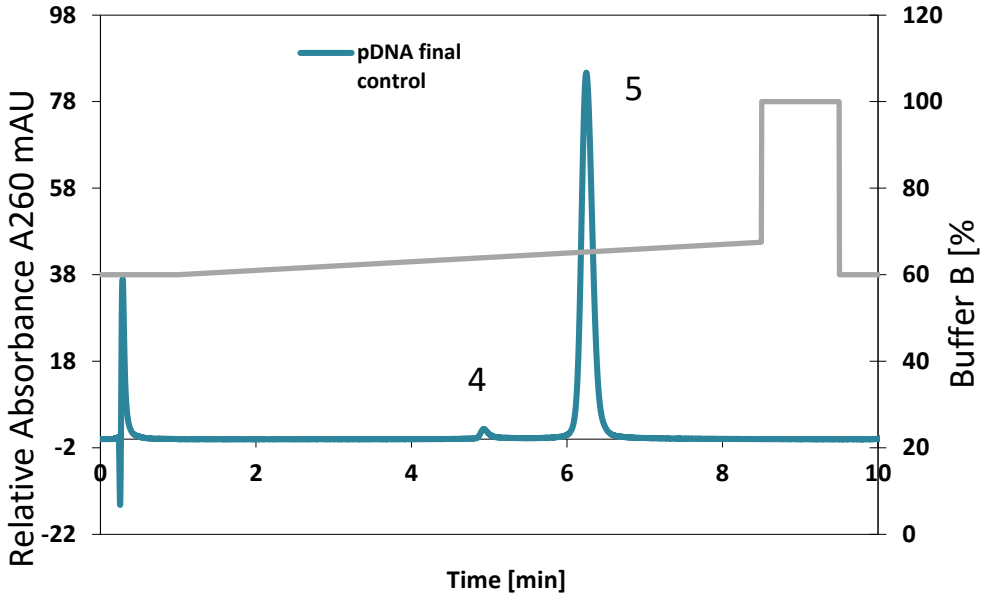
Adjustment with $(NH_4)_2SO_4$

↓

CIM® C4

↓

Buffer exchange



Conditions: Flow rate – 1 ml/min; Buffer A – 200 mM Tris pH 8.0 and buffer B – 200 mM TRIS + 1 M NaCl pH 8.0; Injection volume – 5 µl; UV detection – 260 nm; Peak 1 – OC pDNA form; Peak 2 – SC pDNA form;

Topoisomers	
OC	2 %
SC	98 %

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CIMmultus™ Plasmid Process Pack purity results

CIM DEAE Step

- RNA and proteins are separated from pDNA
  - RNA free
  - Host cell proteins free

CIM C4 HLD Step

- sc DNA separated from
  - oc DNA
  - genomic DNA free
  - Endotoxins free

	Process After C4 HLD Step
Homogeneity (SC pDNA)	≥ 97%
Host cell DNA – removed	> 99.5%
Host cell proteins - removed	> 99%
Endotoxin	< 2 EU/mg pDNA
RNA - removed	> 99%
*At the end buffer exchange is needed	

CIMmultus™ Plasmid Process Pack recovery results

	Alkaline lysate	CIM DEAE-8	CIM C4-8
pDNA (µg/ml)	28	630	300
pDNA (mg)	40	38	34
Homogeneity (% SC)	94	95	98
Endotoxins (EU/mg pDNA)	200	12.4	1.1
Host cell proteins (µg/ml)	190	30	11
gDNA (µg/mg pDNA)	20	10.3	3.4
RNA (µg/ml)	N.D.	0	0
Yield (%)	**100%**	**95%**	**90%**

Economic benefit for the customer using CIM® Monolith Plasmid DNA purification pack

1 ml CIM monolith – BIA Sep

Calculations	
Buffer	76,3 ml buffer/mg pDNA
Time	23,6 min /mg pDNA
Recovery	85%
Costs using column for 1 run	
Quantity of purified pDNA	5,10 mg PDNA
€ (Column costs)	114 €/mg pDNA
€ (Column + buffer)	114 €/mg pDNA
Costs using columns for 10 runs	
Quantity of purified pDNA	51 mg pDNA
€ (Column costs)	11,4 €/mg pDNA
€ (Column + buffer)	11,8 €/mg pDNA
Costs using columns for 20 runs	
Quantity of purified pDNA	102 mg pDNA
€ (Column costs)	5,7 €/mg pDNA
€ (Column + buffer)	6,1 €/mg pDNA
€ (column + buffer+ work)	15,4 €/mg pDNA

CIM monolithic columns offer **3 times cheaper** purification costs of pDNA for gene therapy

Particle based

Calculations	
Buffer	108,0 ml buffer/mg pDNA
Time	70,0 min /mg pDNA
Recovery	79%
Costs using column for 1 run	
Quantity of purified pDNA	4 mg PDNA
€ (Column costs)	227 €/mg pDNA
€ (Column + buffer)	228 €/mg pDNA
Costs using columns for 10 runs	
Quantity of purified pDNA	40 mg pDNA
€ (Column costs)	23 €/mg pDNA
€ (Column + buffer)	24 €/mg pDNA
Costs using columns for 20 runs	
Quantity of purified pDNA	79 mg pDNA
€ (Column costs)	11 €/mg pDNA
€ (Column + buffer)	12 €/mg pDNA
€ (column + buffer+ work)	42 €/mg pDNA

CIMmultus™ Plasmid Process Pack easy scale up

	Bed volume	pDNA capacity
CIM-1	1 ml	6 mg
CIM-8	8 ml	48 mg
CIM-80	80 ml	480 mg
CIM-800	800 ml	4.8 g
CIM-8000	8000 ml	48 g

1st generation RNA Industrial Purification Platform Process

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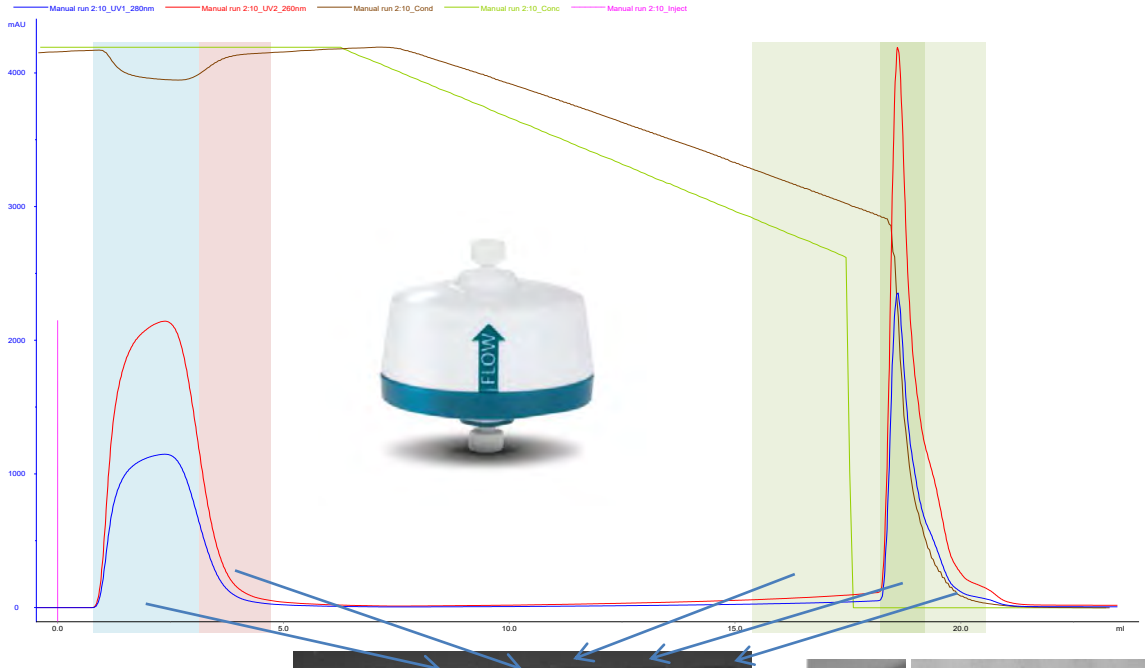
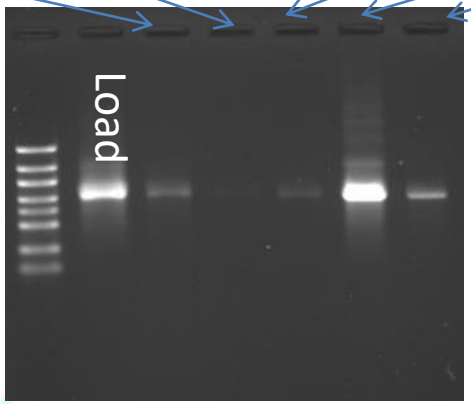
mRNA purification:

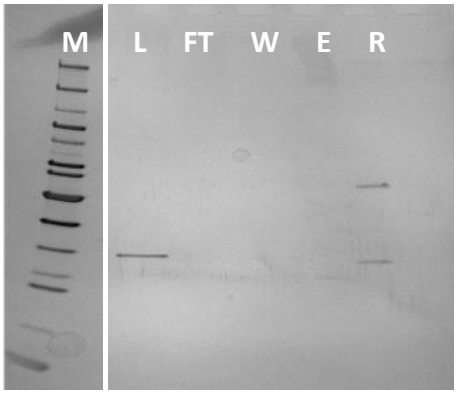
Purification of mRNA on C4 HLD 1 ml column in lin-step gradient



Experimental conditions:

- Mobile phase A: 50 mM TRIS + 10 mM EDTA + 1.75 M NaCl, pH 7.2
- Mobile phase B: 50 mM TRIS + 10 mM EDTA, pH 7.2
- Flow rate: 1 mL/min
- Gradient: combination of linear (0%-cca.35% B) and step gradient
- Regeneration with 2-propanol
- Load: ~1 mg IVT mix Luc mRNA (mRNA material 10x diluted prior loading)





Fraction	mRNA (%)
Flow through	21.8
Wash	7.7
Pre-elution	15.3
Elution	**60.4**
Elution tail	5.1
Total	110.4

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2nd generation AAV Industrial Purification Platform Process

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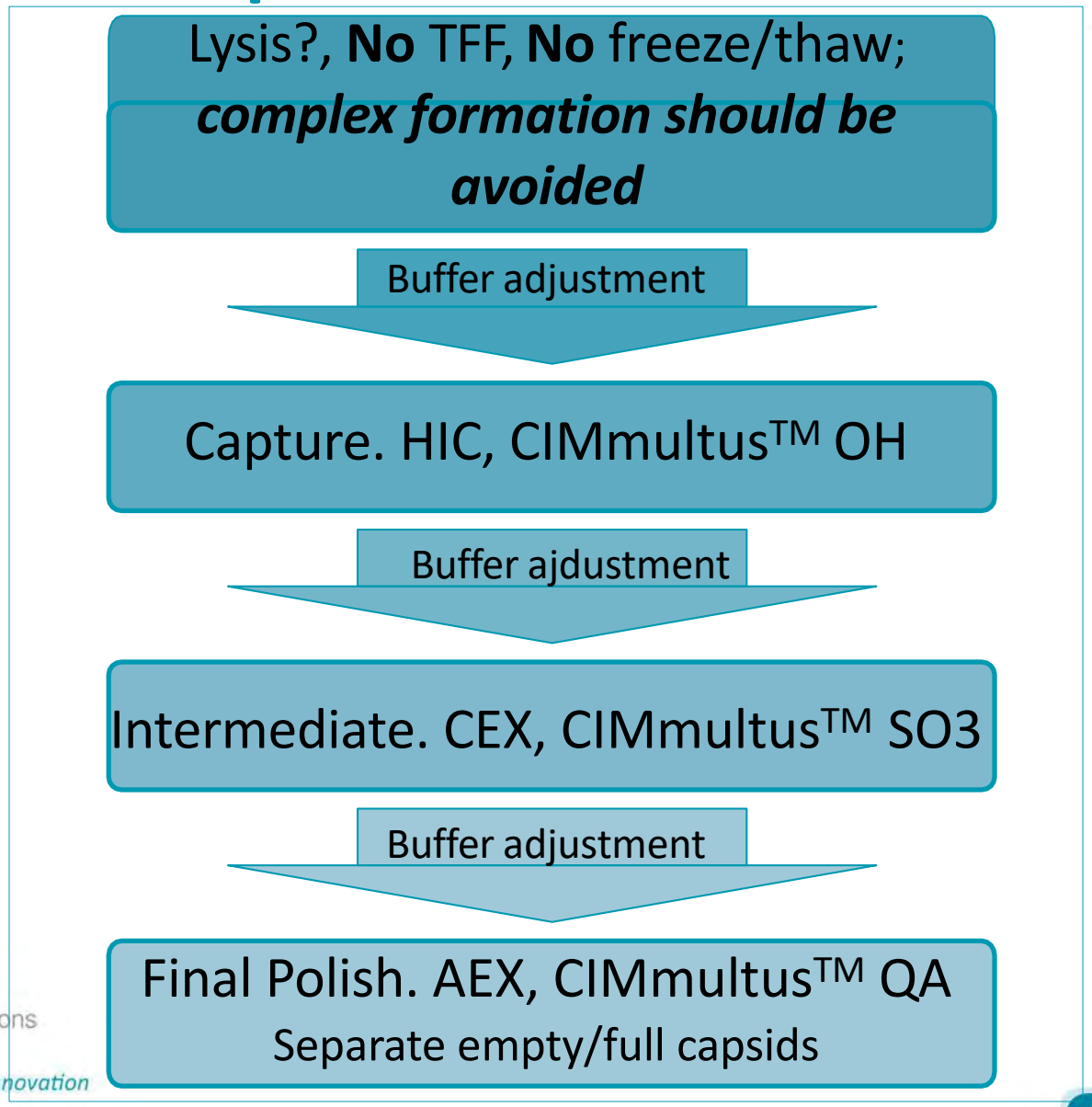
2nd generation – works for all AAV serotypes, for extracellular produced viruses with >80% yields



>80% yield from harvest to fill and finish for extracellular AAV with low amount of dead cells

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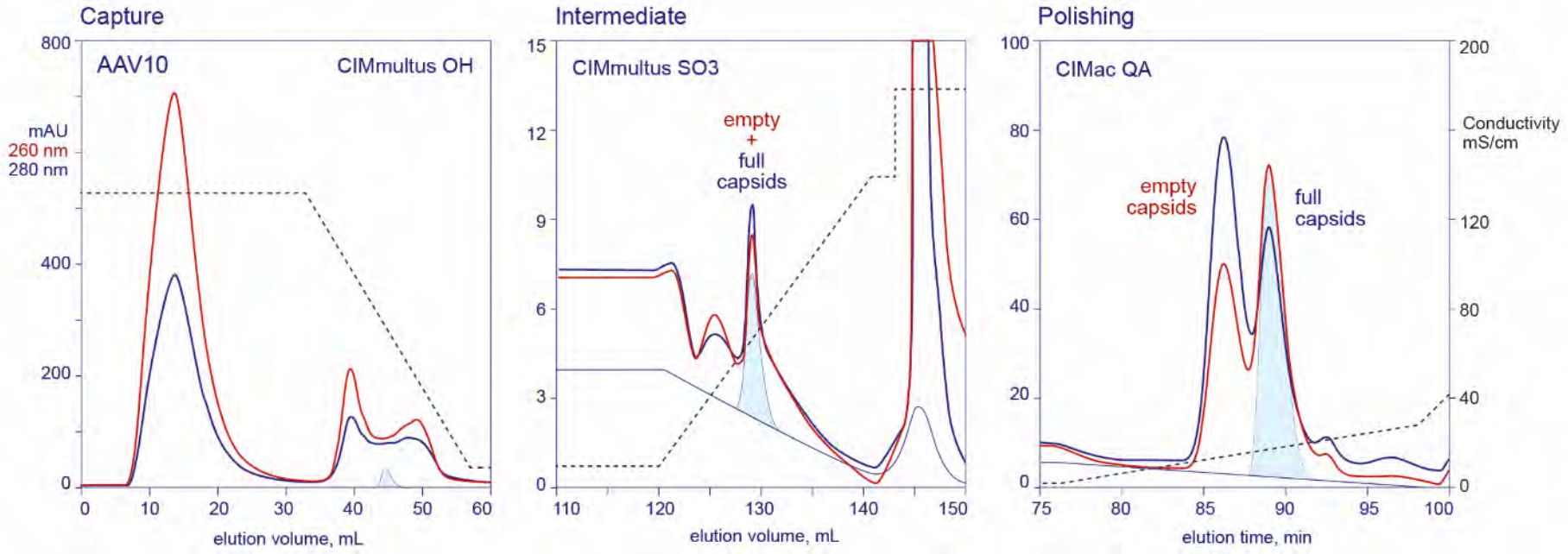
2nd generation process - non-affinity AAV process for all AAV serotypes



1. The sample is diluted 1:1 and loaded directly to the OH column to minimize shear and process time. **Removal of highly charged proteins and RNA/DNA**.
2. The OH fraction is diluted and loaded directly to the cation exchange column. **Removal of highly charged proteins**.
3. The cation exchange fraction is diluted and loaded directly to the anion exchange column to **separate empty/full and remove residual impurities**

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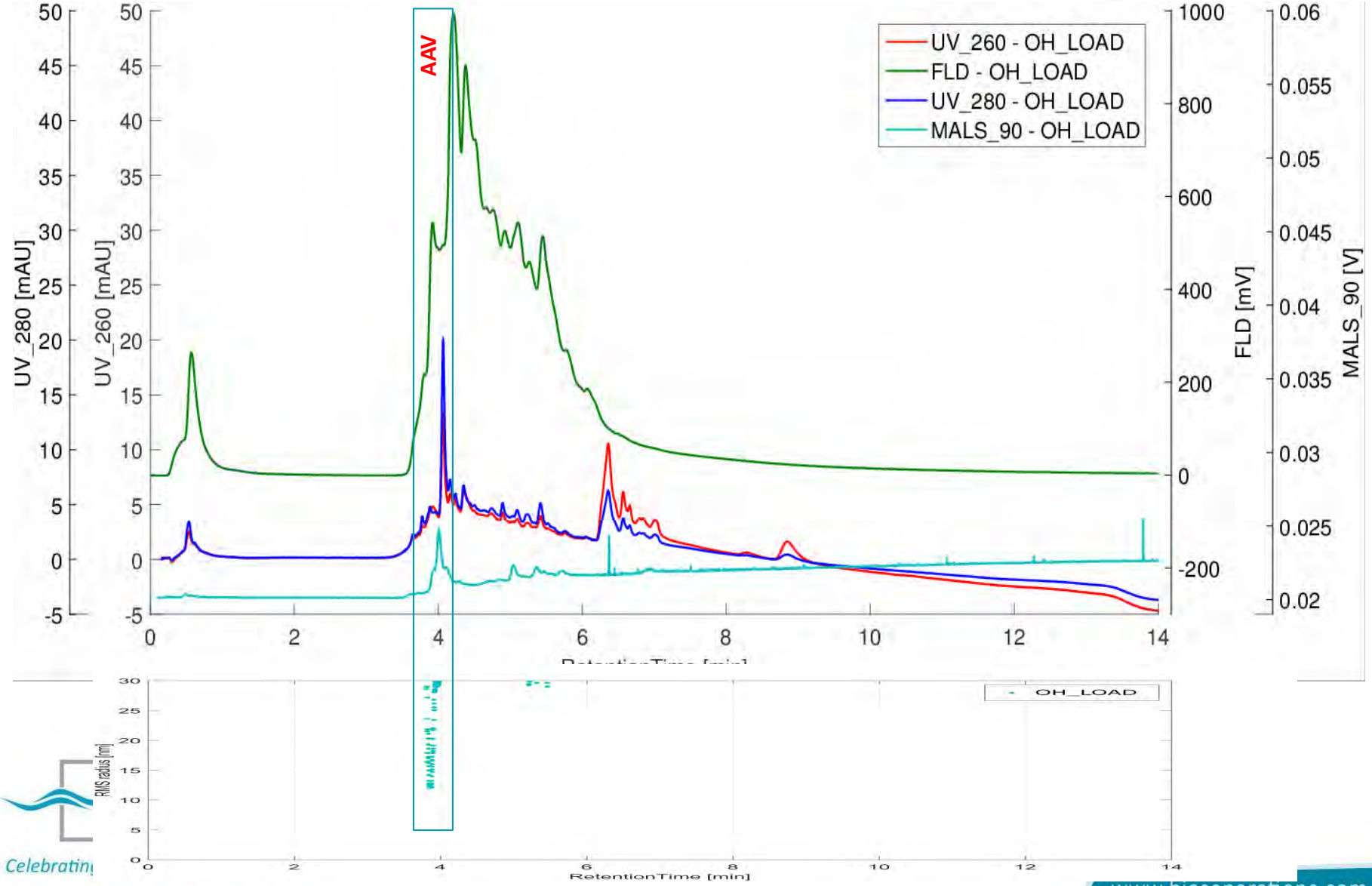
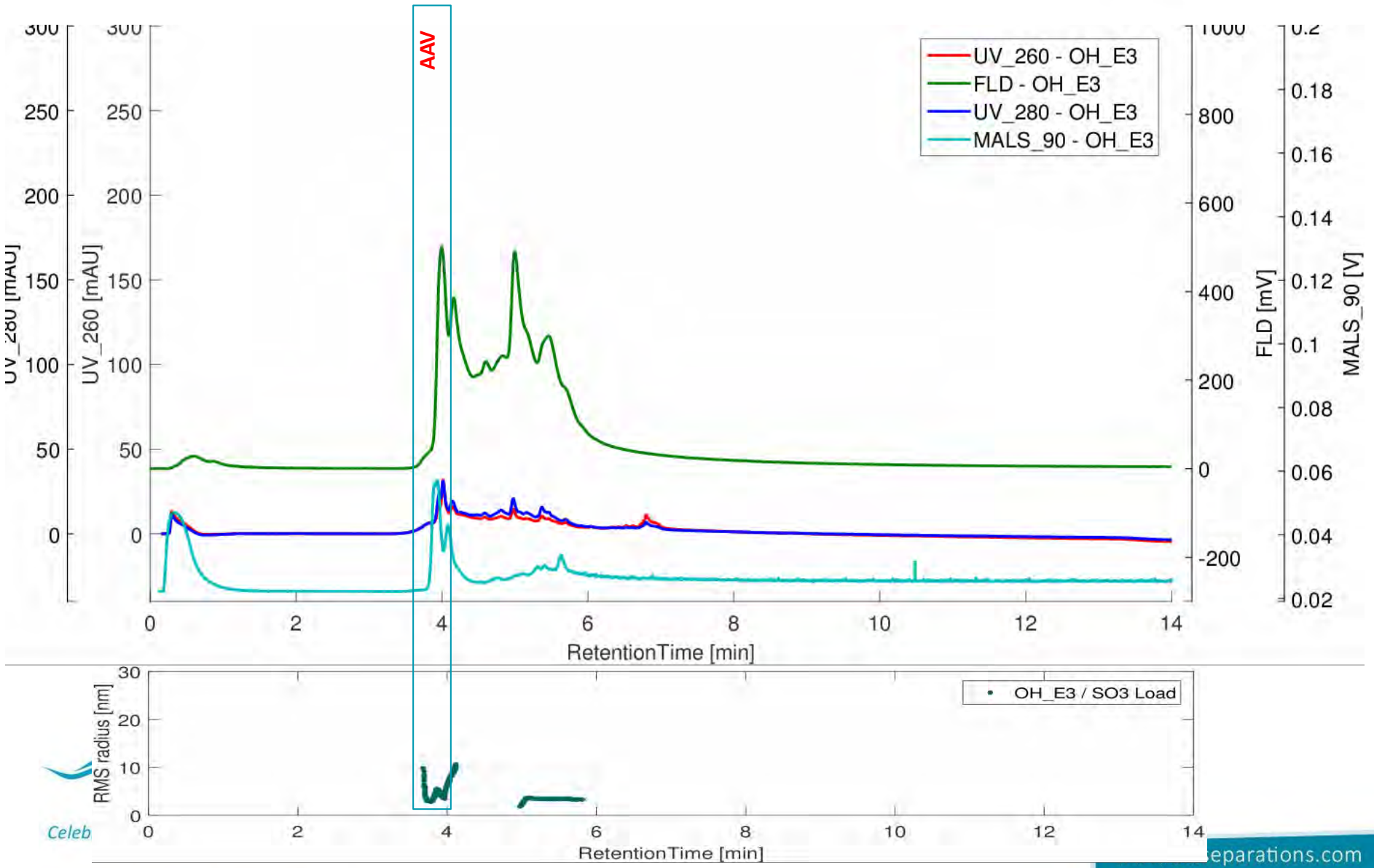
Thanks to Stephen M. Kaminsky and Hyunmi Lee, Belfer Gene Therapy Core Facility of Cornell University, New York and Nicole Brument, INSERM UMR 1089, Université de Nantes, France.

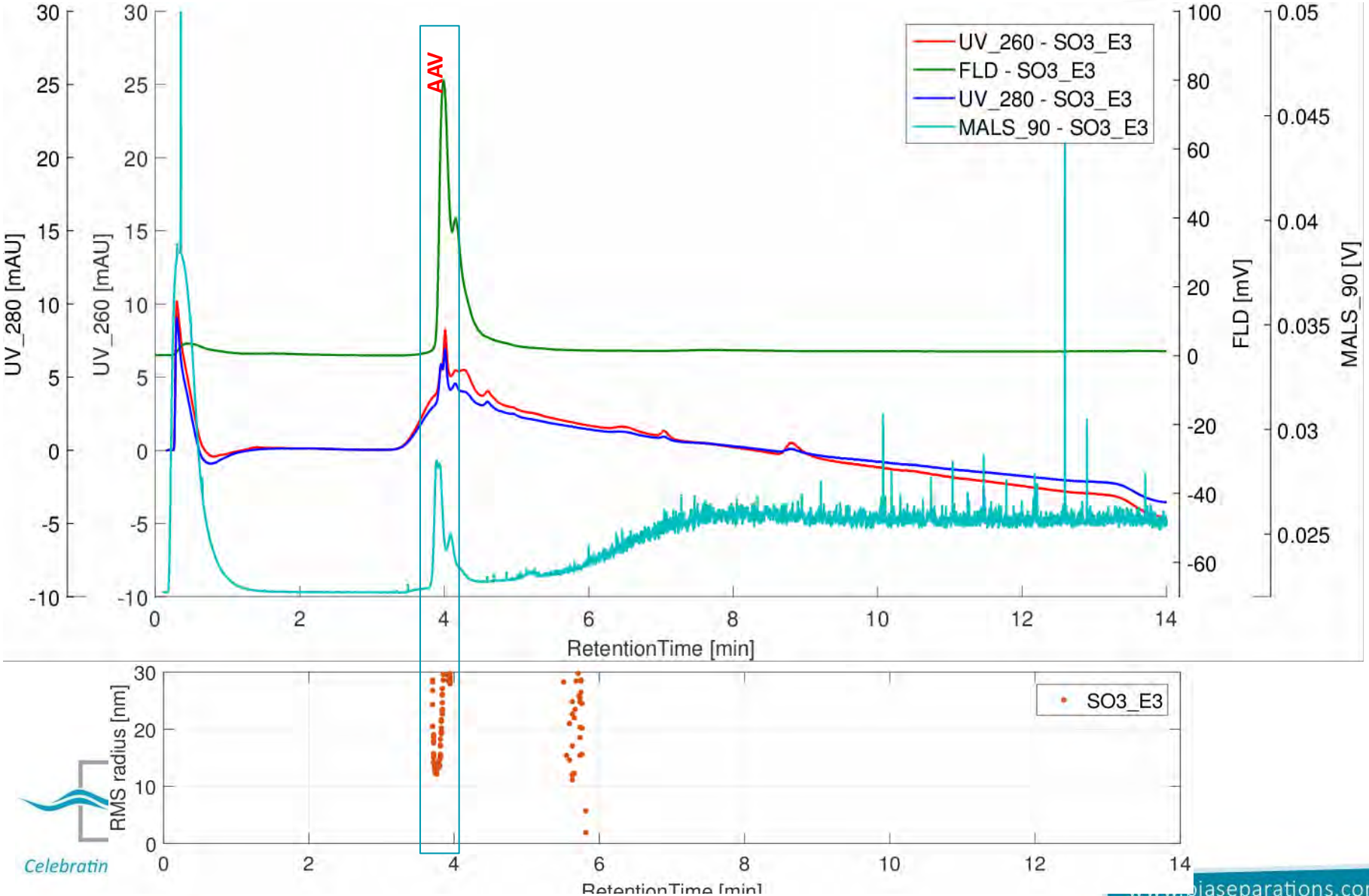
Fast process development using CIMac QA column in liner gradient: *CIM OH step - Load*



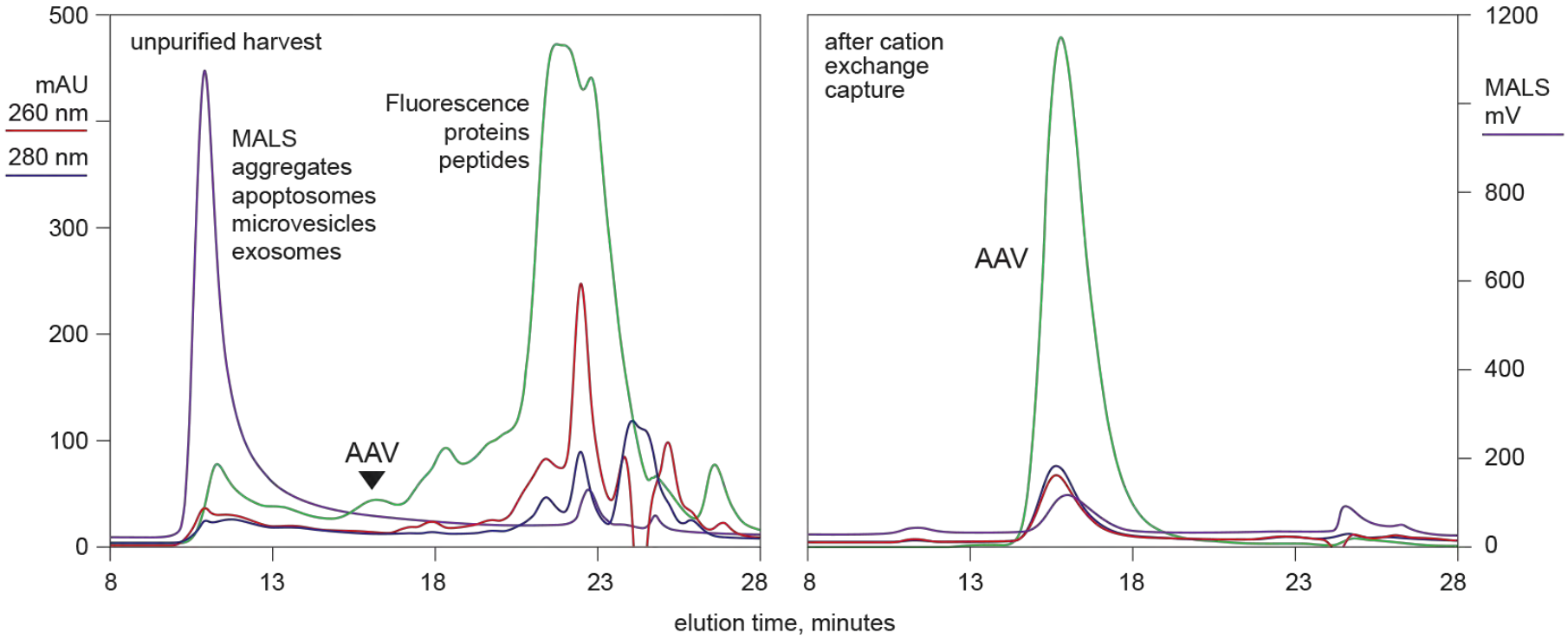
Fast process development using CIMac QA column in liner gradient: *CIM OH step – AAV elution fraction*



Fast process development using CIMac QA column in liner gradient: *CIM SO3 step – AAV elution fraction*



Complexes are the source of residual DNA/RNA in the product - Need for orthogonal analytical methods: *Characterization by analytical SEC*



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.

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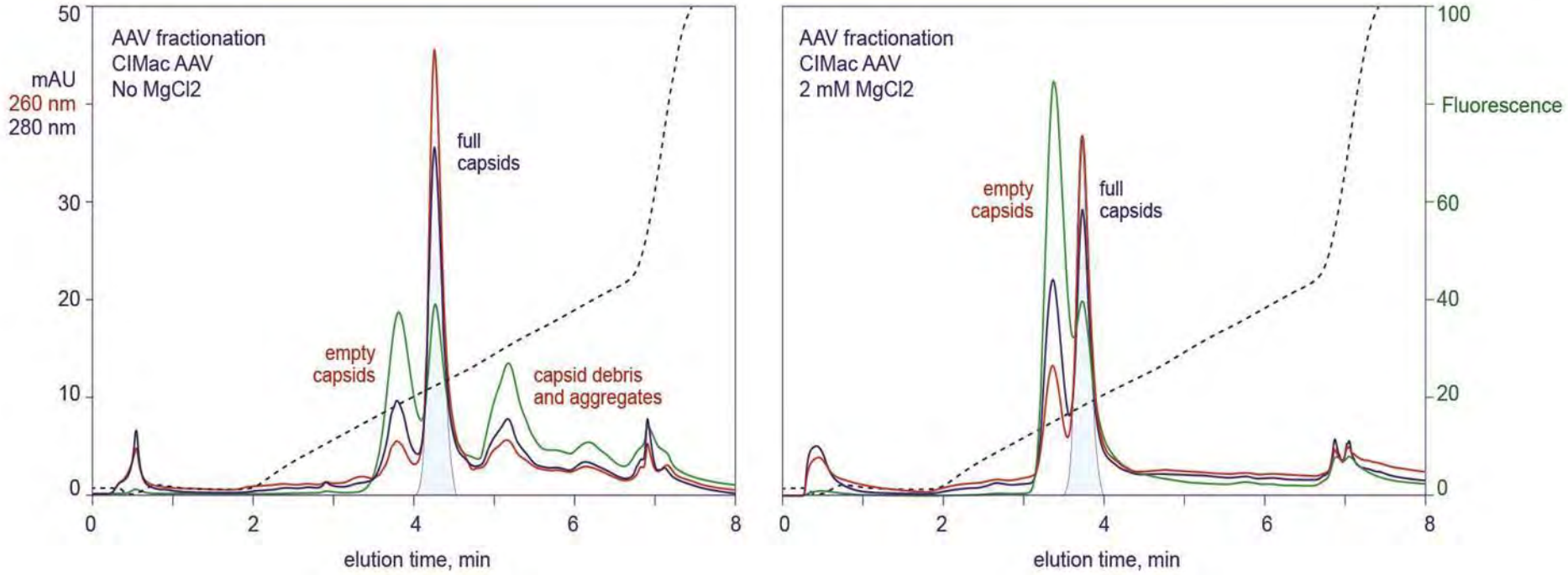
Thanks to Stephen M. Kaminsky and Hyunmi Lee, Belfer Gene Therapy Core Facility, Department of Genetic Medicine, Weill Medical College of Cornell University, New York.

Rapid discrimination of empty and full capsids using PATfix with CIM QA column accelerates process development, validation, and in-process control

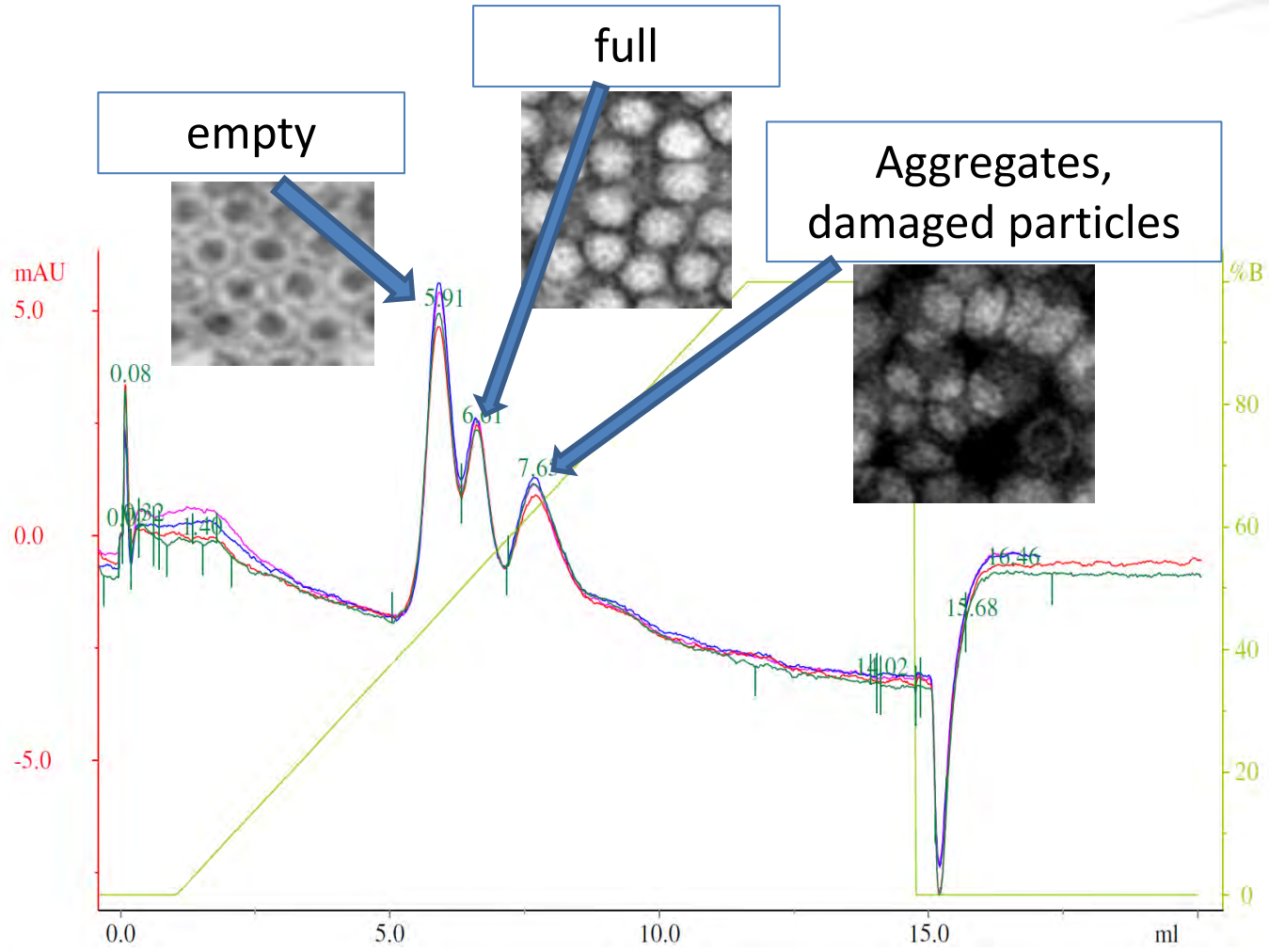


Excess magnesium ions are necessary for empty capsid integrity

Full capsids absorb more 260 because of their DNA content. Empty capsids absorb more 280 because of their protein dominance. Tryptophan-tyrosine fluorescence also emphasizes protein content but gives higher sensitivity than UV absorbance.

Thanks to Stephen M. Kaminsky and Hyunmi Lee, Belfer Gene Therapy Core Facility of Cornell University, New York and Nicole Brument, INSERM UMR 1089, Université de Nantes, France

Separations of empty, full and damaged AAV capsids using Anion exchange CIMmultus™ QA column



Damaged particles usually found after affinity step. Using SO3 might need to be used before QA to get good empty/full separation

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Empty/Full AAV determination – for accurate results orthogonal methods are requested

WORK IN PROGRESS:

AAV serotype	client/partner	sample	cryoTEM Partner A	cryoTEM Partner B	AUC	HPLC (UV, FLU, MA)
AAV10	Partner 1	before QA				
AAV2/8	Partner 2	QA LOAD/QA E				
AAV9	Partner 3	QA LOAD/QA E				
new partners welcome						

Influence of the serotype, source (cell line), method used, purity, residual DNA/RNA,… on the E/F result.

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3rd generation AAV Manufacturing Platform Process Development – towards ultra low residual DNA/RNA

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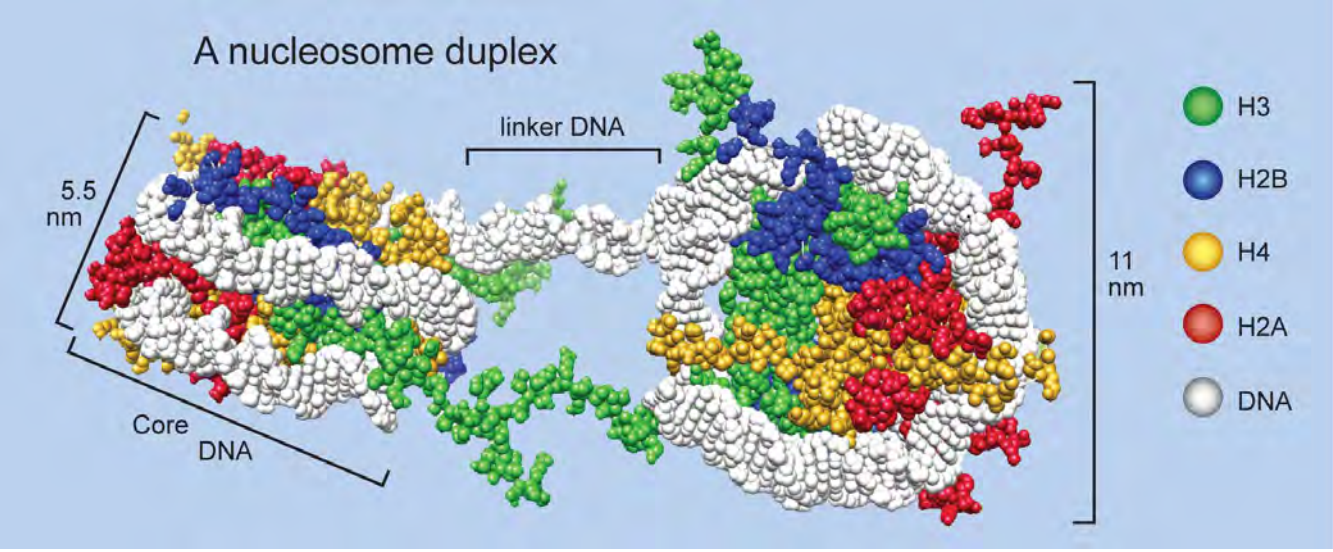
Key task to reach ultra low residual DNA/RNA is proper management of Chromatin structures

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

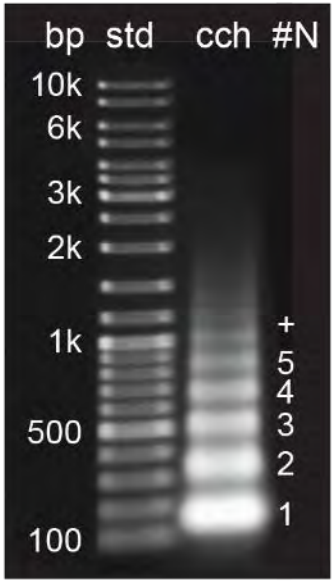


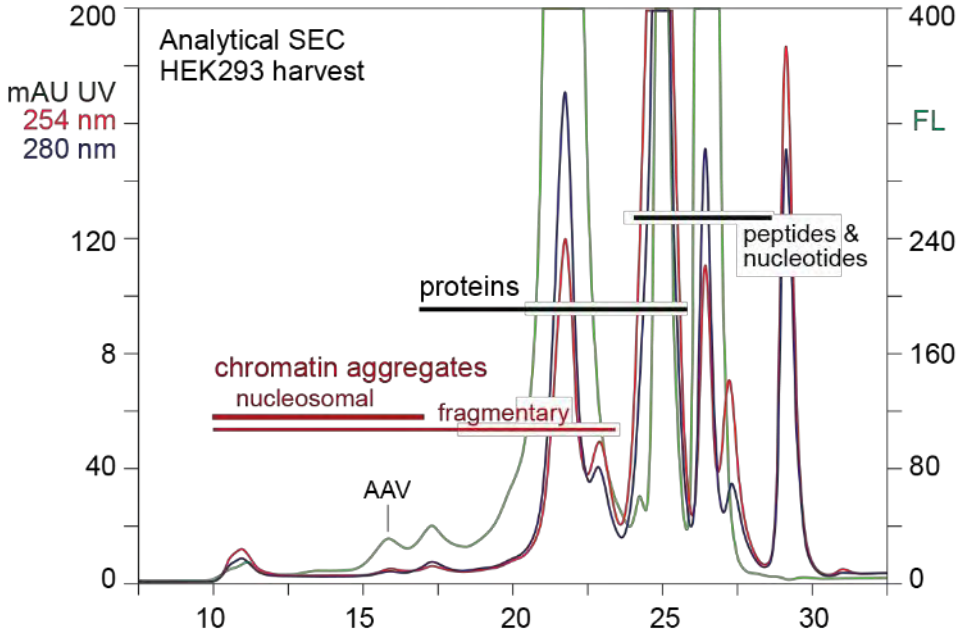
Histones are extremely hydrophobic and highly positively charged, with isoelectric points ranging from 9 to 11. DNA has a pK of about 2.6.

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 persists for months in the cell culture and stick to the product

Agarose stained with GelRed™ and analytical size exclusion chromatography.





Chromatin in cell culture harvests is commonly highly degraded, consisting mostly of short arrays containing from 1 to 10 or more nucleosomes.

It exists in two primary forms: 1) **nucleosomal aggregates** contain at least one nucleosome. 2) **fragmentary chromatin aggregates** consist of individual histone proteins and very small DNA fragments.

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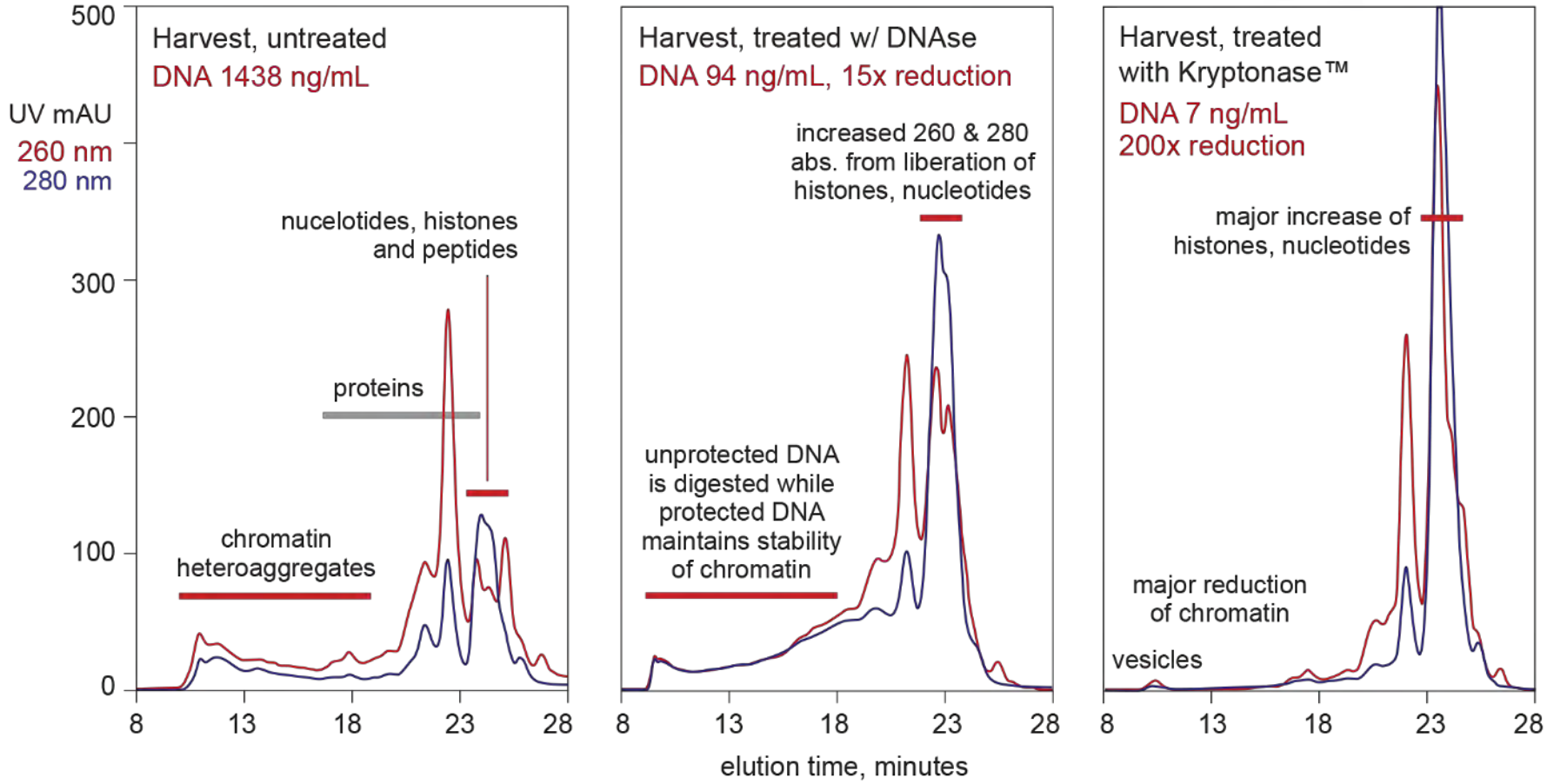
Chromatin and nucleosome removal is the key task of the 3rd generation AAV manufacturing process



Analytical size exclusion chromatography using UV detector 260 nm (red) and 280 nm (blue).

Kryptonase™ treatment – patent pending

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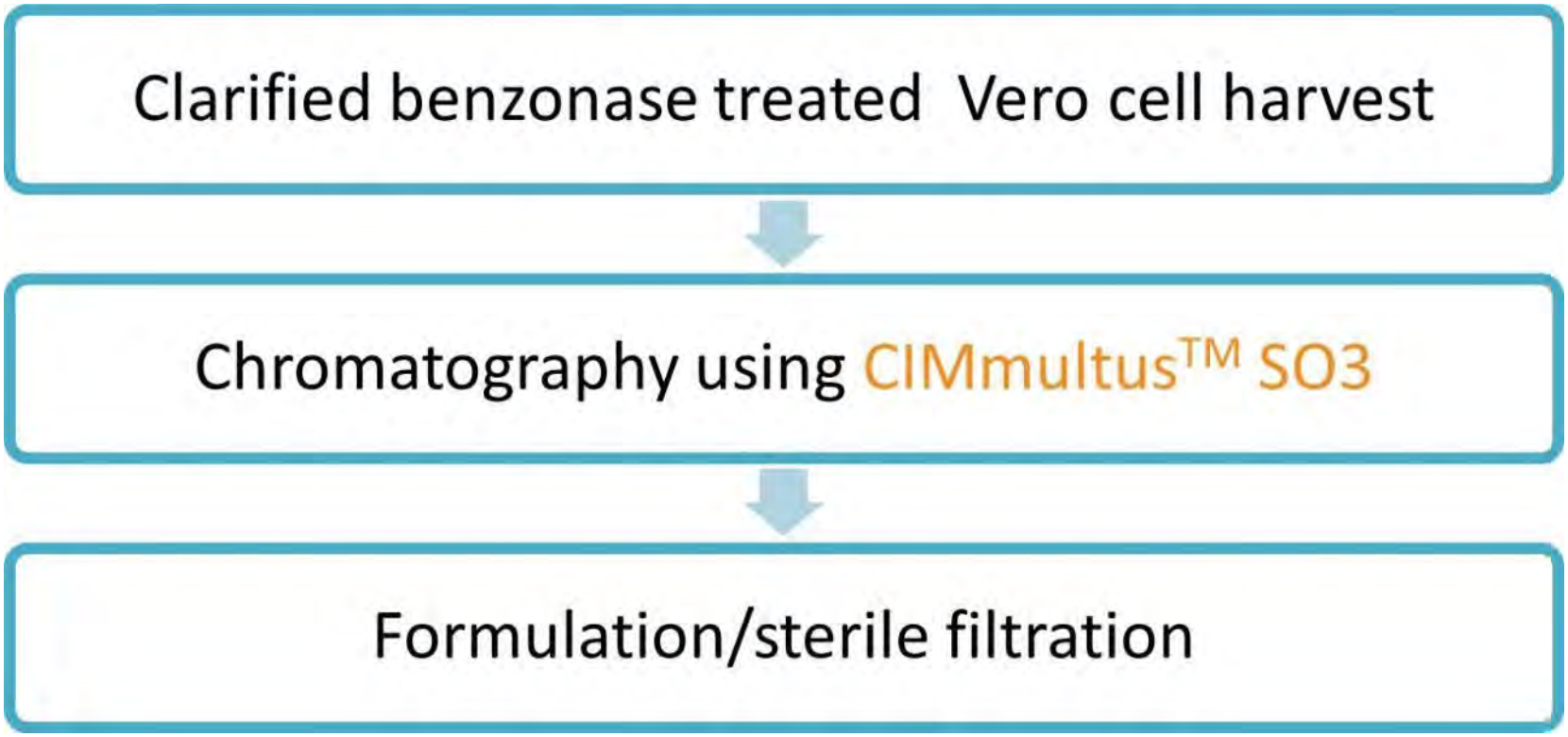
One-step monolith purification of oncolytic influenza virus produced in Vero cells

Flow-chart of the process (goal: fast and cheap process to produce oncolytic vaccines)*

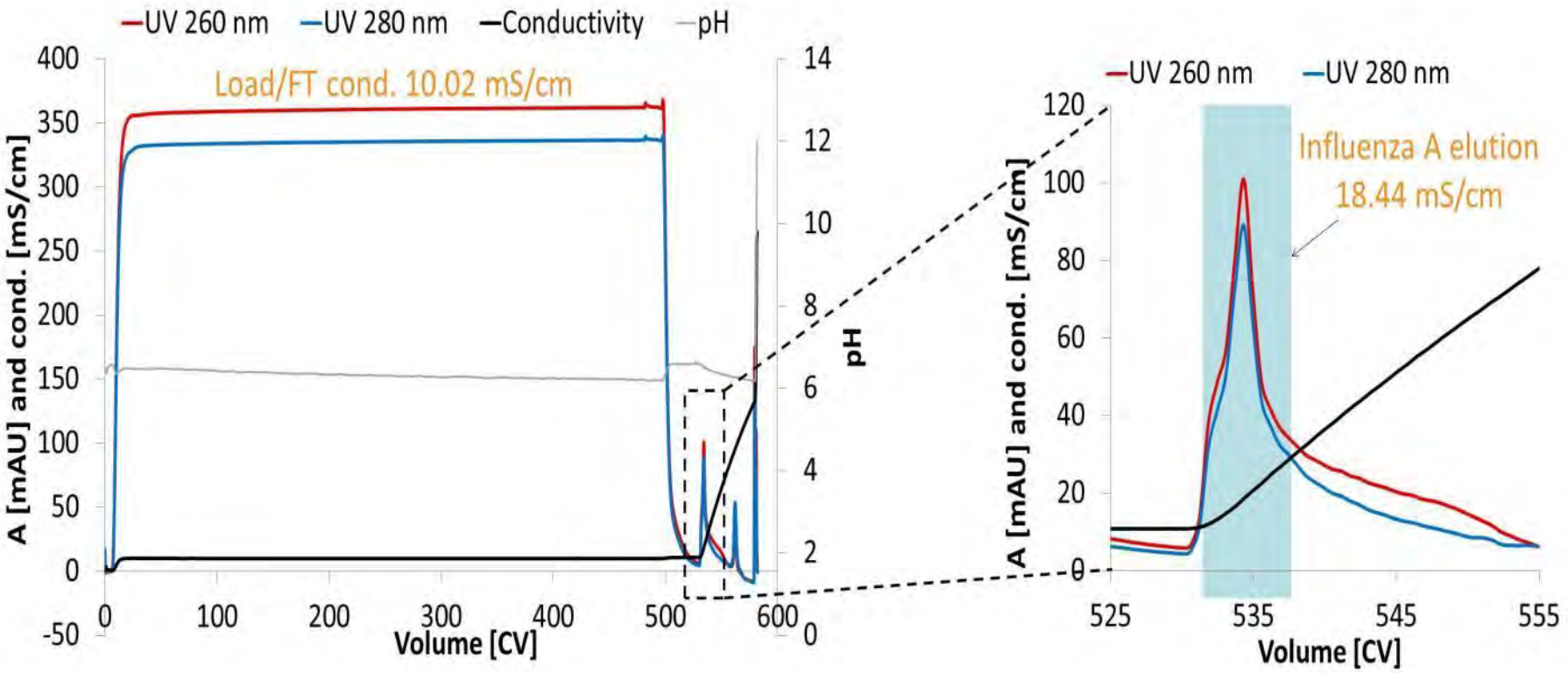


*Patent pending

Chromatographic conditions

- Centrifuged cell culture harvest containing 1E+7–1E+9 influenza virus particles per mL was treated with benzonase, diluted 1.5-fold with HEPES-**sucrose** buffer and applied without further treatment to a monolithic column (**no freeze/thaw**!!)
- Column: CIMmultus™ SO3-1 Advanced Composite Column (Pores 2 um)
- Sample dilution / column equilibration buffer A: 50 mM HEPES, 200 mM sucrose, pH 7.0
- Column equilibration buffer B: 50 mM HEPES, 200 mM sucrose, 2 M NaCl, pH 7.0
- Elution buffer A: 50 mM HEPES, 200 mM sucrose, 100 mM NaCl pH 7.5
  Elution buffer B: 50 mM HEPES, 200 mM sucrose, 2 M NaCl pH 7.5
- Flowrate: 5 ml/min; Detectors: UV 260, 280, 700 nm; Conductivity, pH
- Analytical assays: hemagglutination (HA), Focus forming assay (FFA), Bradford, Picogreen, total Flu HPLC PATfix™

Chromatography using CIMmultus™ SO3



Chromatogram of Influenza A purification using CIM multus SO3 column and zoom-in to the main fraction. Virus elutes at 18.44 mS/cm under neutral pH conditions.

Advantages of the One-step monolith purification of oncolytic influenza virus produced in Vero cells

- **VERO cell based production of Influenza viruses enables omitting Tangential flow filtration (TFF) prior to the chromatography step – reduces costs, operating time and improve overall process yields and purity.**
- **Overall process yield are more than 80% due to less complex and aggregate formation during the DSP (no TFF, no freeze/thaw).**
- **DNA and host protein contamination reduced by > 95%.**
- **50 times higher concentration of infective virus particles than in the original cell culture.**
- **Dilution to achieve the target virus concentration is required only to prepare the final formulation.**

Platform purification of exosomes from cell cultures

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Platform purification of exosomes from cell culture

Monoliths are ideal for purification of exosomes.

Multi-Angle Light Scattering (MALS) combined with UV absorbance enables simultaneous in-line characterization of exosomes and contaminating proteins.

Cell Culture Harvest

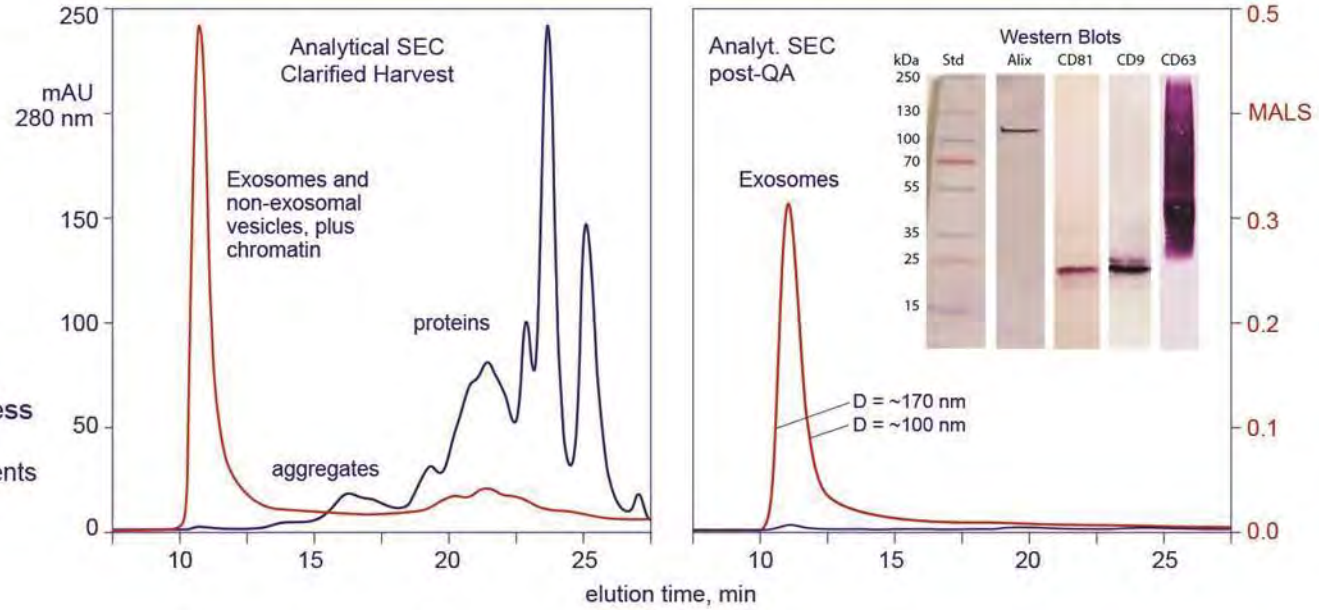
CIMmultus OH

BIA FTx*

CIMmultus QA

Scalable 1-day process

*novel proprietary elements patents pending



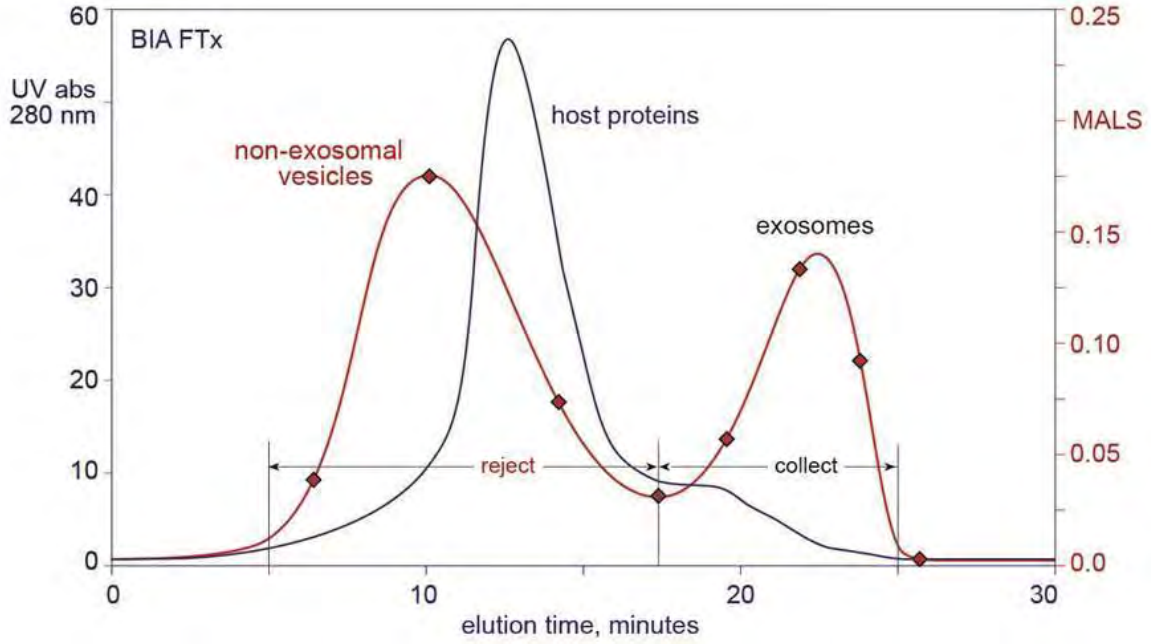
MALS also enables exosome size determination of highly purified preparations. FTx removes non-exosomal vesicles. QA separates different exosome populations. Immunological confirmation by at least three markers is essential.

Thanks to FiberCell Systems Inc. for providing exosome cell culture harvests.

Platform purification of exosomes from cell culture

BIA's new FTx technology removes non-exosomal vesicles.

Non-exosomal vesicles are the most difficult contaminants to remove from exosomes because of their similar size and surface chemistry.

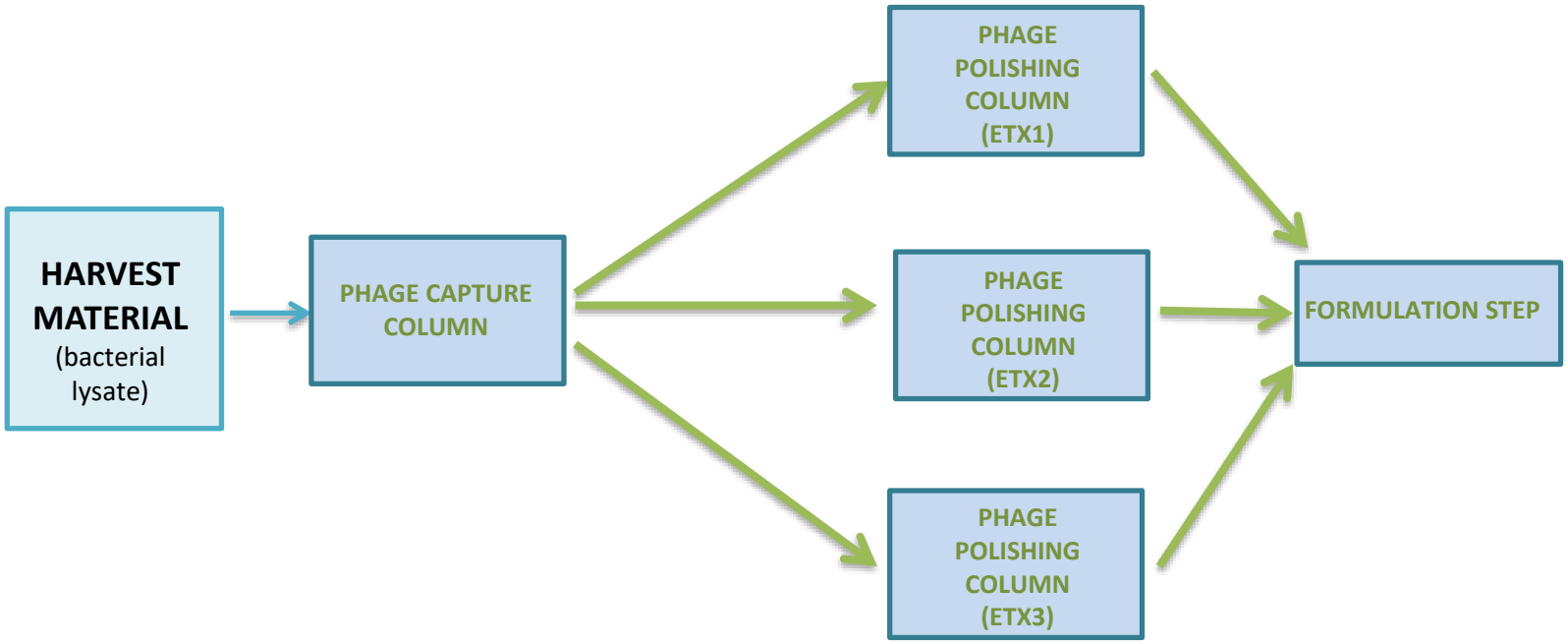


FTx also removes protein contaminants and other agents that could interfere with the final QA step.

Note again that MALS provides essential guidance about distribution of vesicles. UV absorbance by exosomes is extremely low.

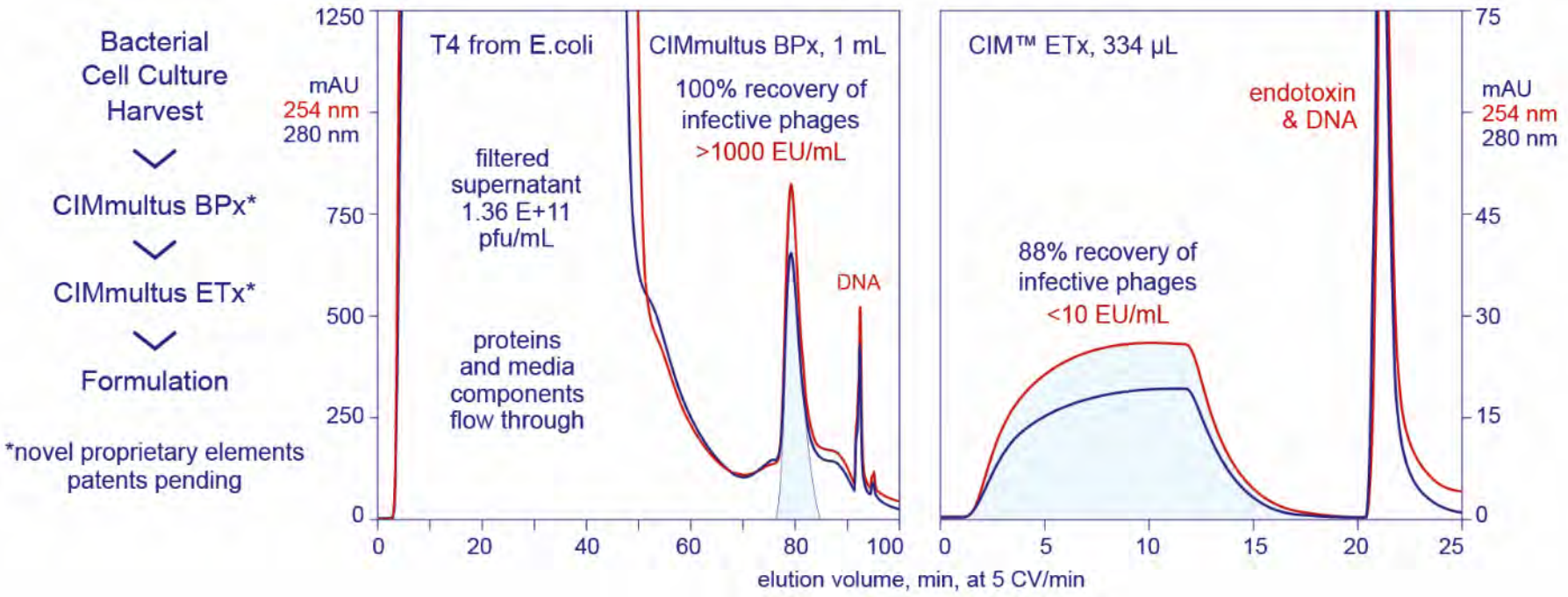
Platform purification of bacteriophages

First step same for all phages, second if endotoxin removal needed, 3 alternatives to be tested



Platform purification of phages

Rapid, high-recovery, two-step platform purification of bacteriophages from gram negative bacteria with less than 10 EU per 1E+10 pfu in about 3 h.



Harvest dilution to direct capture without TFF, then dilution to direct flow-through for the polishing step, both at flow rates of 300 CV/h. Consistent results with all bacteriophages from gram negative bacteria tested to date.

Endotoxin results obtained with EndoSafe™ from Charles River Laboratories.

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BIA Separations - industry standard for production of Gene Therapy, Phage, Vaccines, Exosomes,.. products

- Platform processes for pDNA, AAV, Flu and Adeno, ...
- First drug purified using CIM® monoliths on the market, one passed CPIII trial, 5 projects in CPIII.
- More than 150 projects in CPI – CPII trials (various Influenza, various Adenovirus, various AAVs, bacteriophages, various IgMs, Inter-alpha-inhibitors, exosomes,...).
- More than 1000 projects in pre-clinical trials (Influenza A and B virus (eggs, Vero and MDCK cells), Rabies virus, Rotavirus, AAV, various Adenovirus subtypes, Hepatitis A, Vaccinia, Mulv, MVM, Feline calicivirus, Japanese encephalitis, Crimean-Congo hemorrhagic fever, Hantaan virus, VLP (Hepatitis B, HPV, Influenza, Adenovirus), bacteriophages (Lambda, T4, VDX10, Pseudomonas phage), Tomato and Pepino Mosaic virus, pDNA, IgM, various proteins).

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Thank you for your attention!

ales.strancar@biaseparations.com