

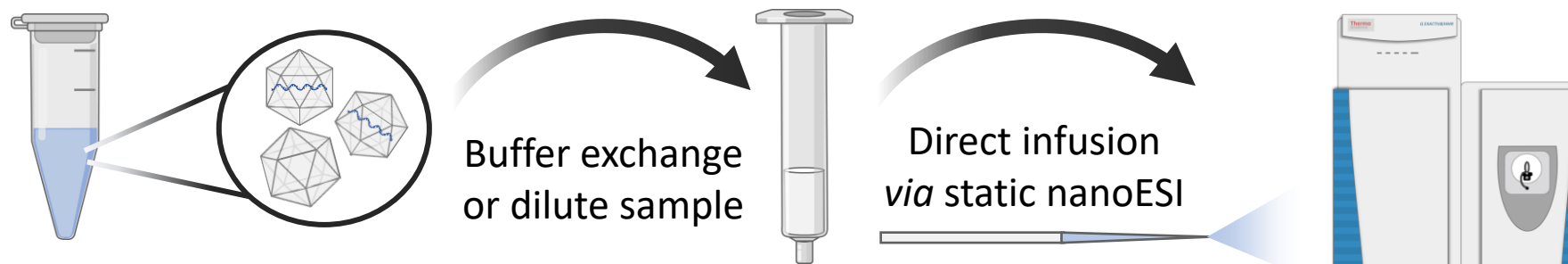
Characterising Viral Vectors for Gene Therapy Delivery Using Mass Spectrometry on Different Levels

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Capsid Fill State Assessment Using Native MS



Thermo Q Exactive UHMR Setting

Resolution 25,000 at m/z 200

Microscans 10

AGC Target 1e06

Max. IT 200 ms

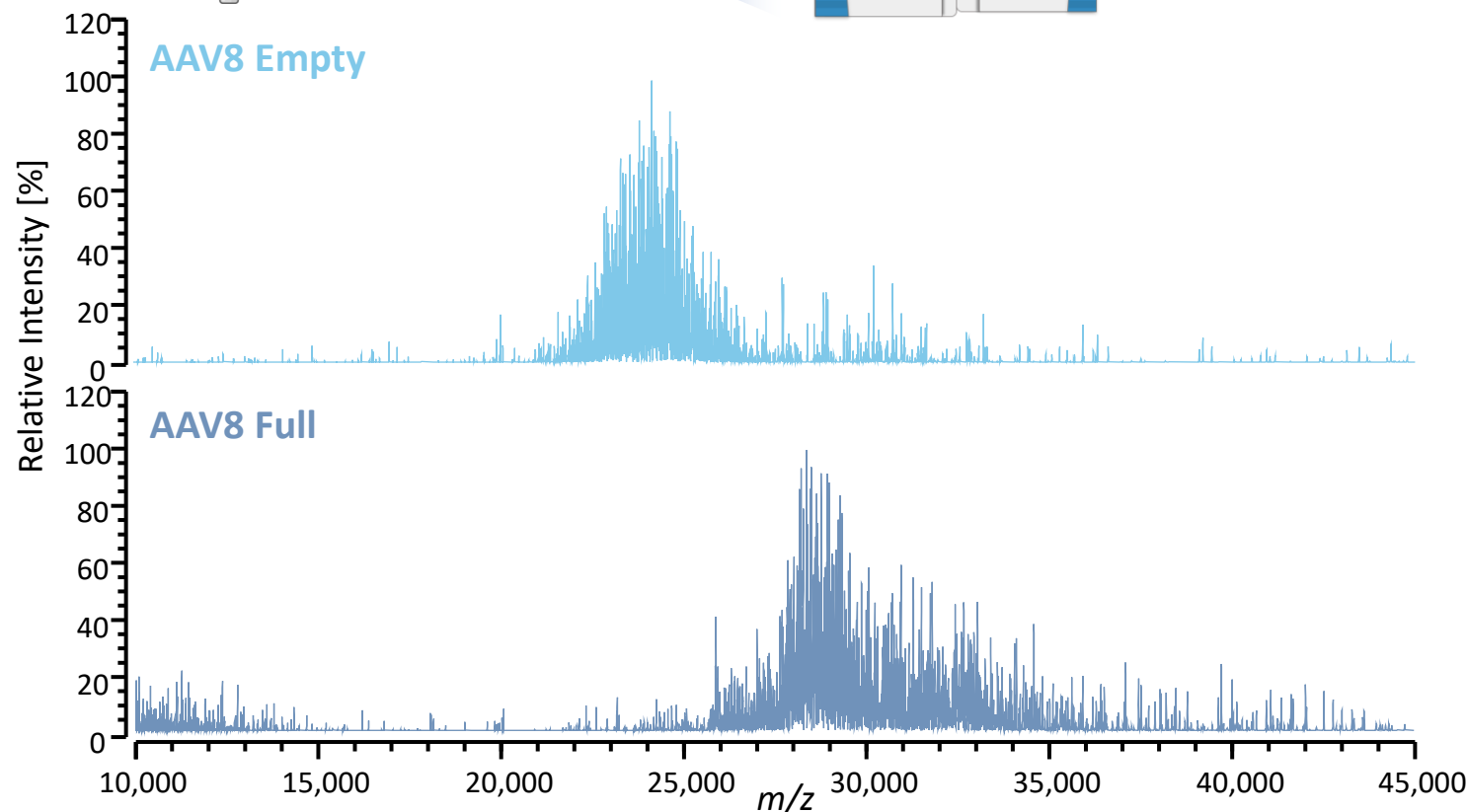
In-source trapping -100 V

Source DC offset -50 V

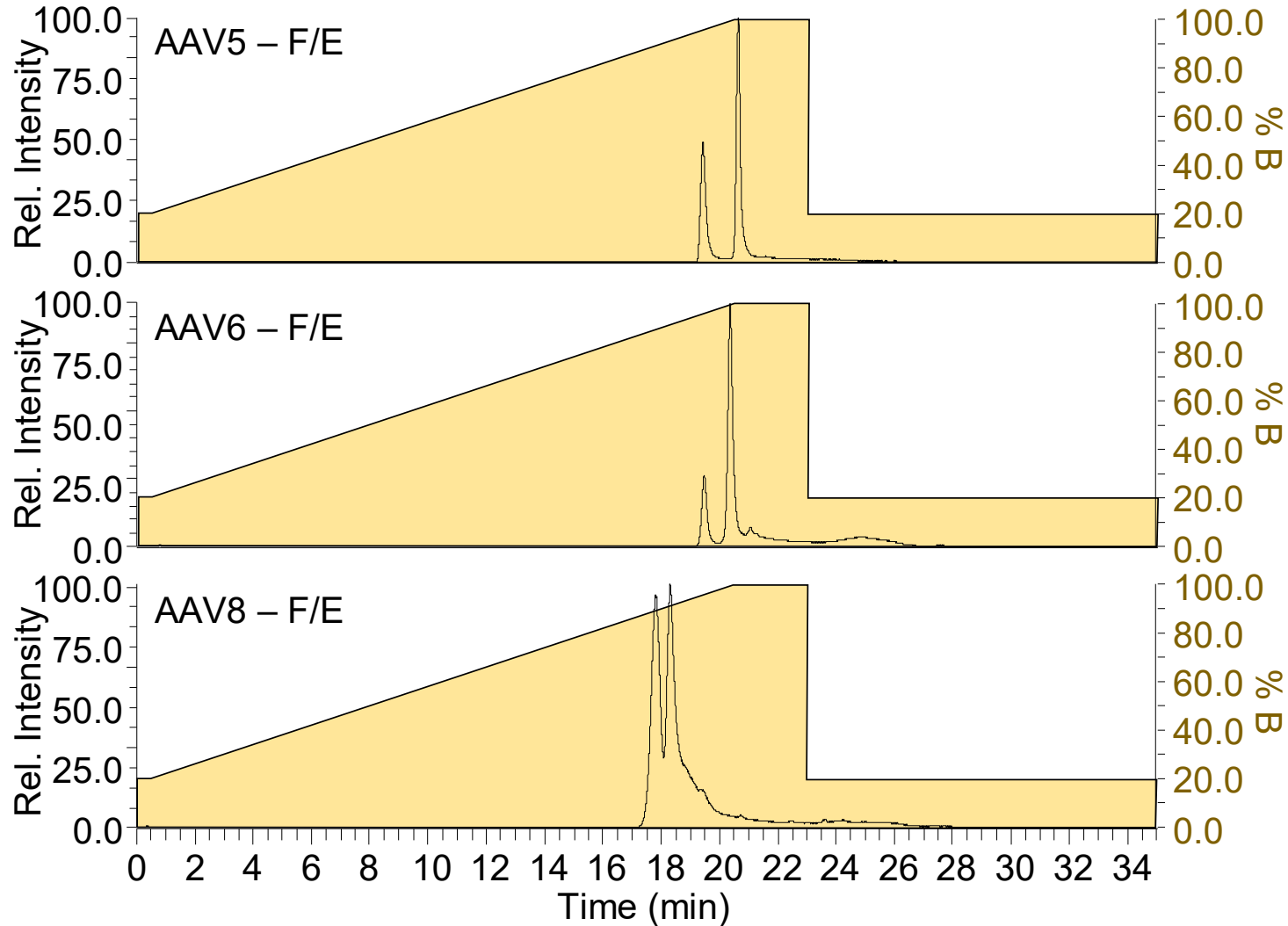
Extended trapping 150 V

Trapping gas SF_6 at 4e-10 mbar

Acquisition 5 mins with transient averaging enabled

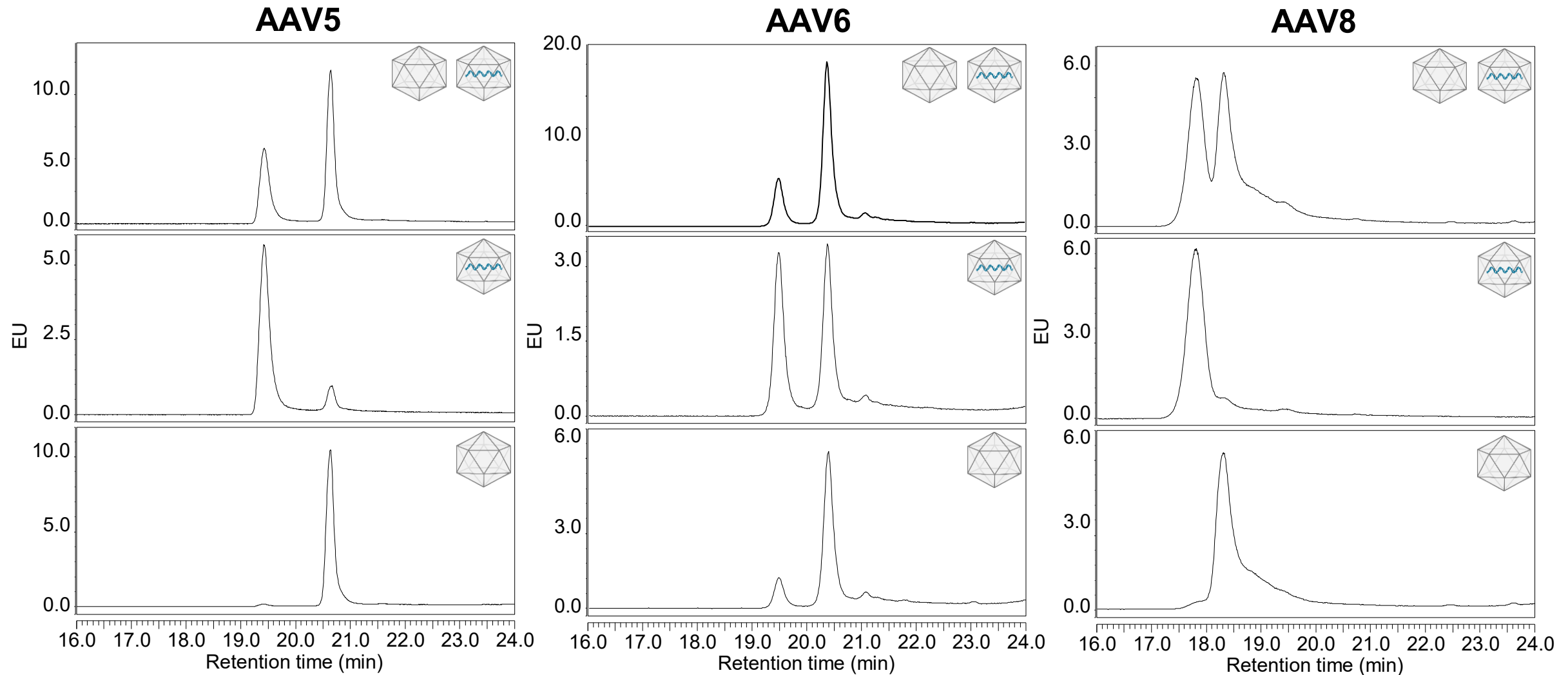


Coupling with Anion Exchange Chromatography

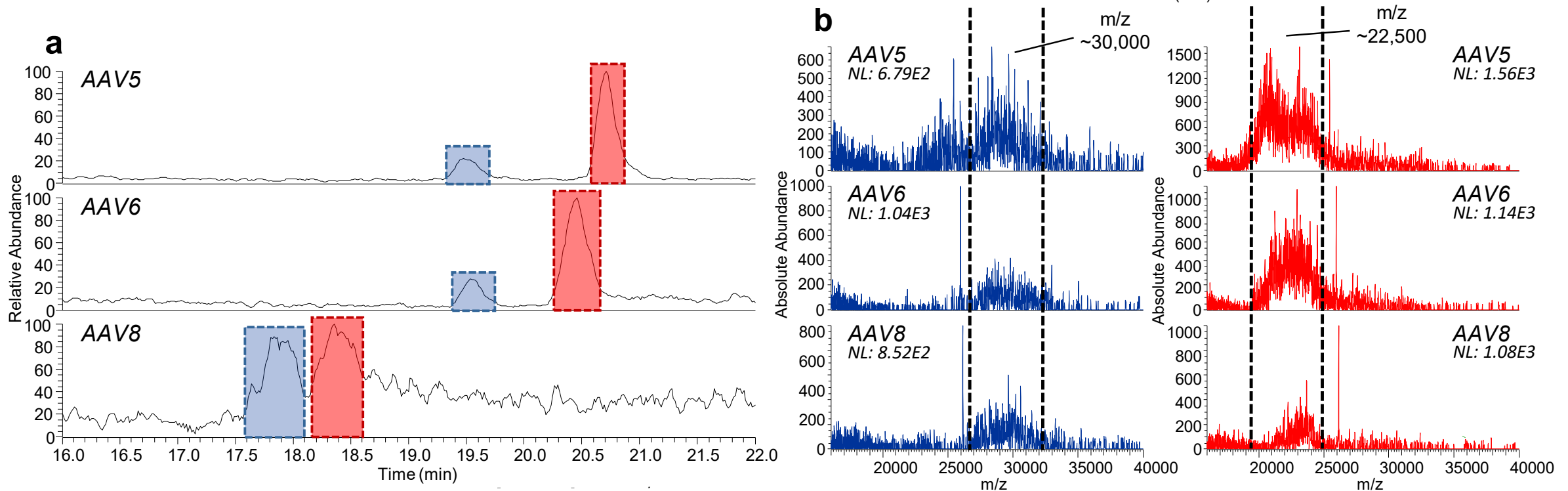


- pH gradient anion exchange separation of full and empty capsids using Thermo Scientific ProPac 3R AEX column.
- Gradient specifically designed to be generic for different serotypes and mass spectrometry compatible.
- pH gradients enable focusing effect, elution occurs when gradient pH = analyte pI, results in sharp chromatographic peaks.

Determination of Capsid Fill State Elution Order

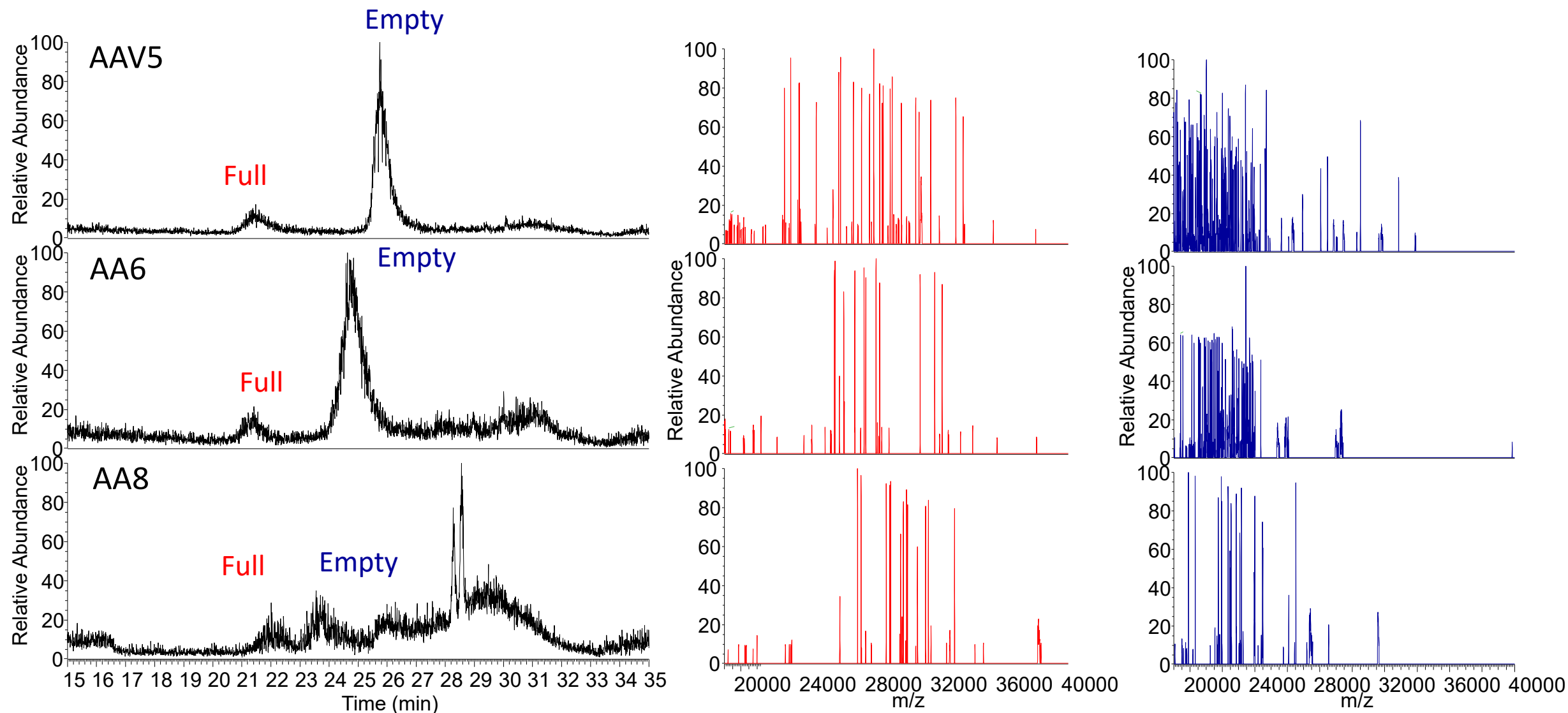


Coupling pH Gradient AEX with Native MS Detection

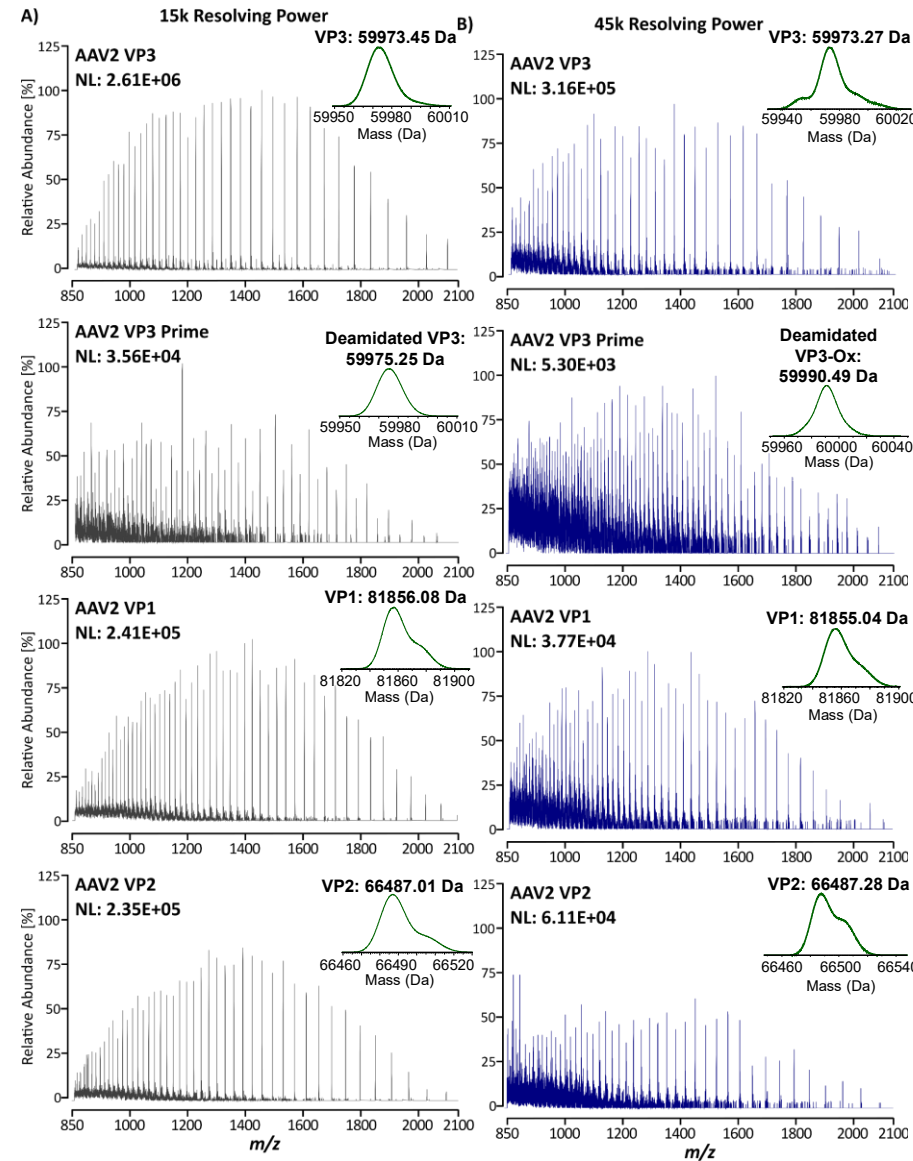
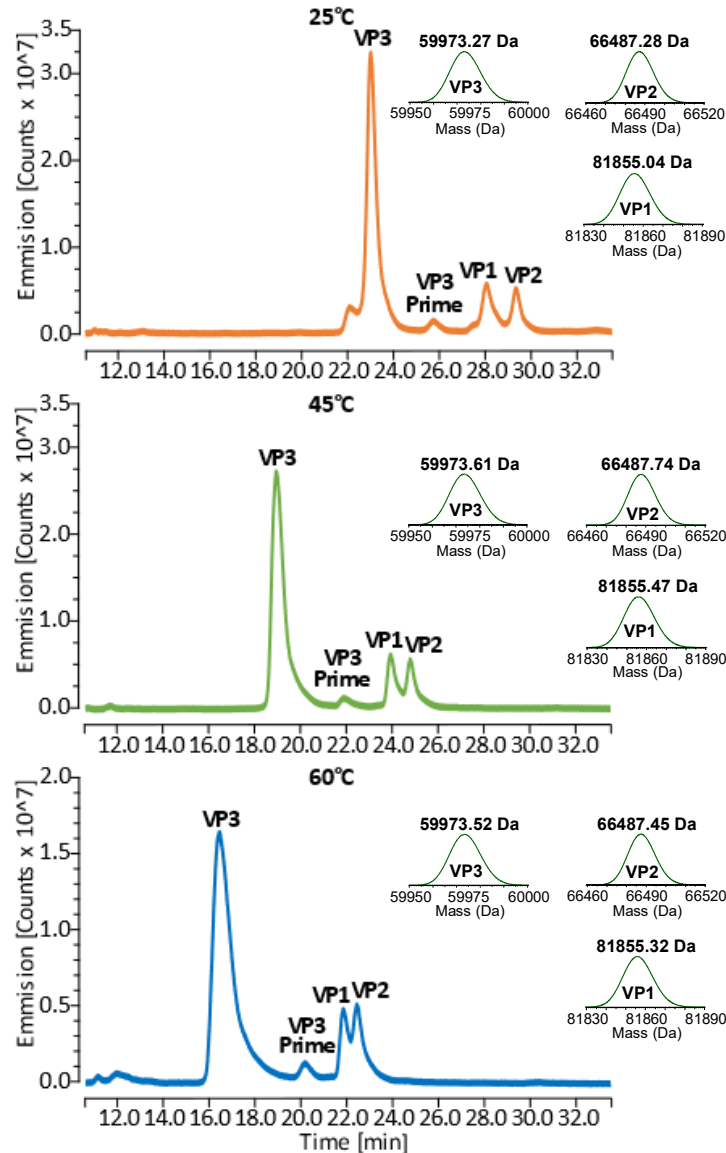


- pH gradient anion exchanged coupled directly to Thermo Scientific Q Exactive UHMR mass spectrometer for confirmation of capsid fill state species identification based on m/z.
- Assuming similar charge, earlier eluting peak contains heavier species explained by the presence of cargo DNA, additional mass of ~0.8 MDa corresponding to CMV-GFP.

Coupling pH Gradient AEX with Charge Detection MS



Viral Protein Separation using LC-MS



- VP separation using hydrophilic interaction LC using an acetonitrile water gradient containing difluoro acetic acid as a mobile phase modifier.
- Fluorescence and MS detection using Thermo Scientific Orbitrap Exploris 240 MS with Biopharma Option.

Method Translation into Rapid Identity Test



Component Identification



BioPharma Finder
Software 4.1

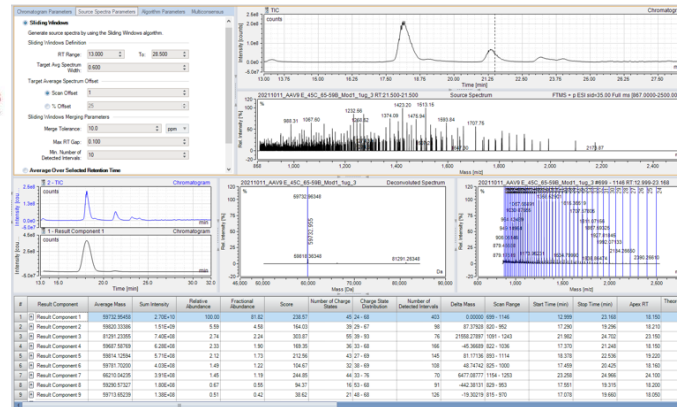


Chromleon 7.3 CDS
No compromise
Processing Method
and Component List
Importation from BPF

Report Generation

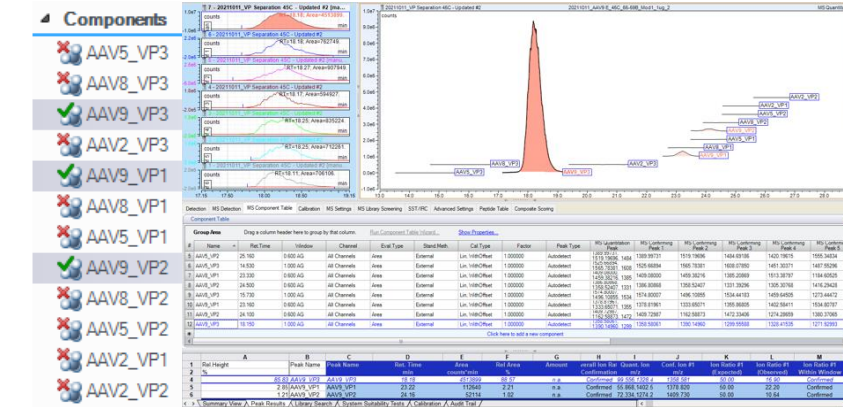


Intact Protein Deconvolution

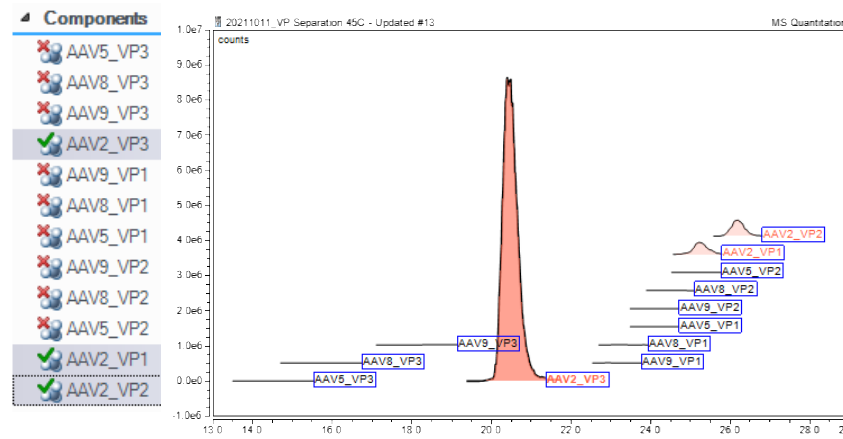


Serotype Profiling

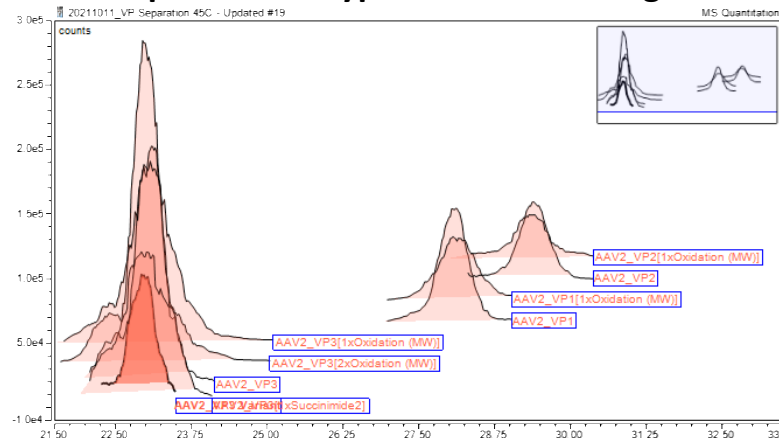
Data Processing Parameter Optimization (Sf9 Derived AAVs)



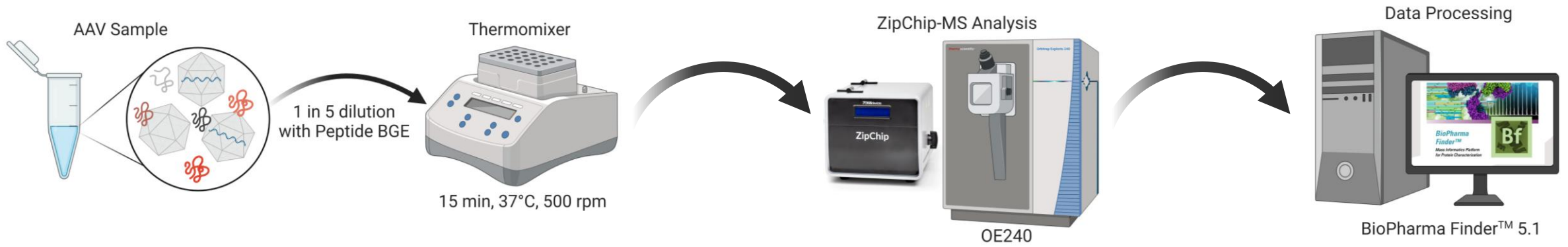
Method Validation (HEK Derived AAV)



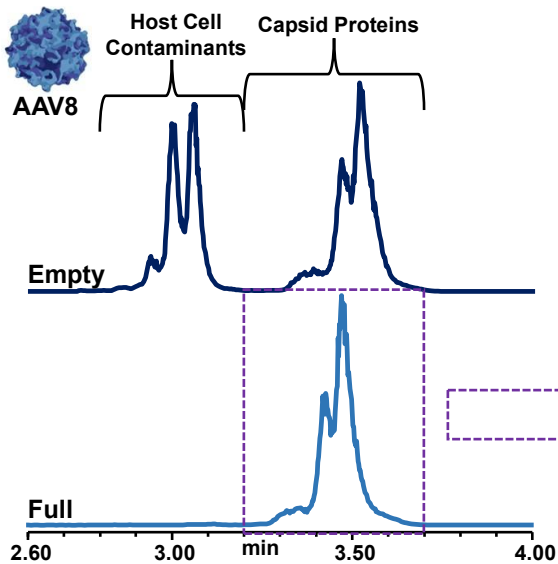
Specific Serotype PTM Monitoring



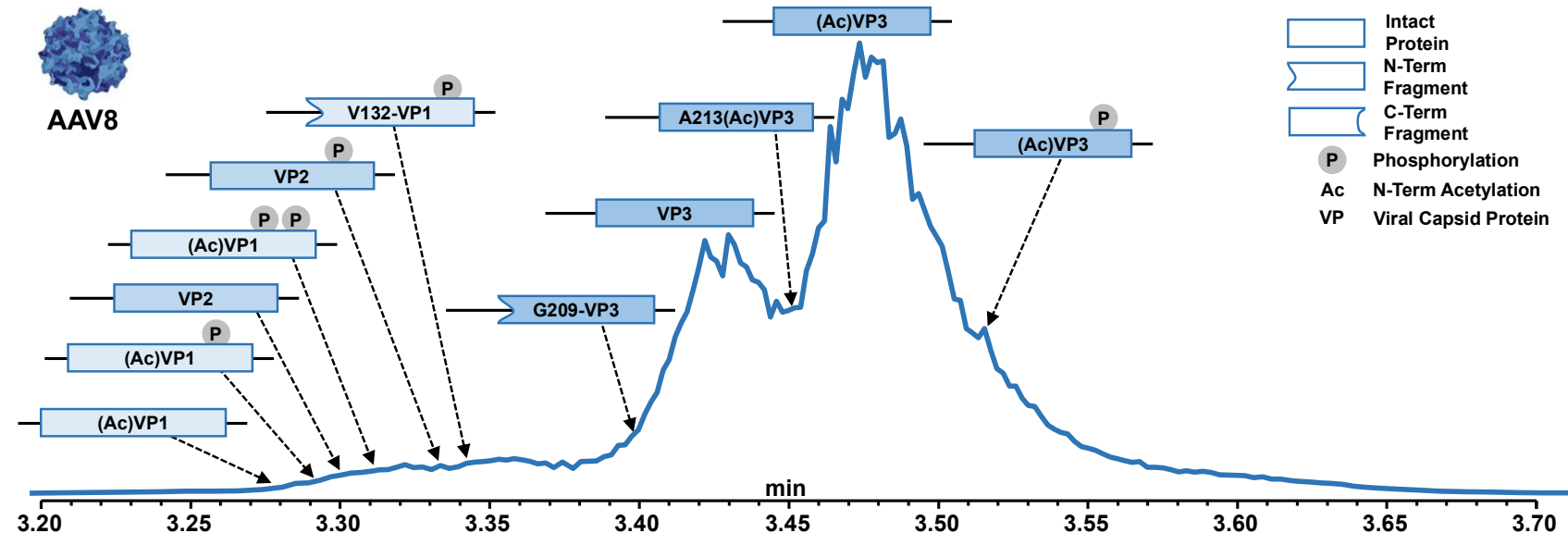
Viral Protein Separation using MCE-MS



Empty vs. Full Profiles



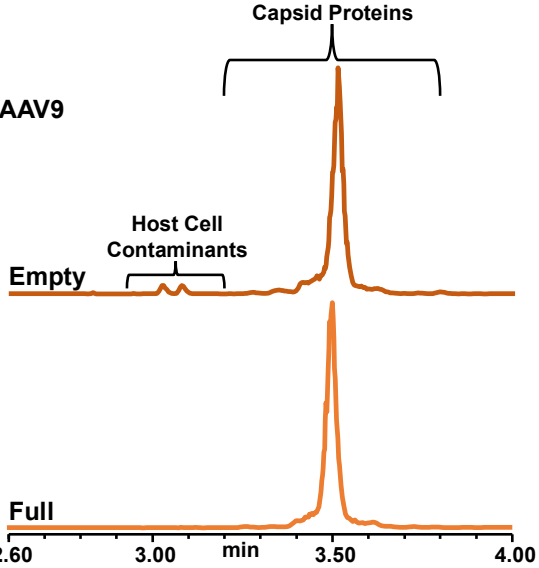
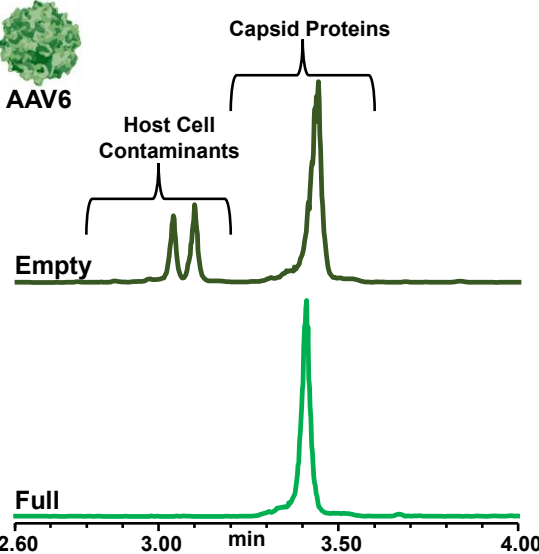
VP Proteoform Characterization



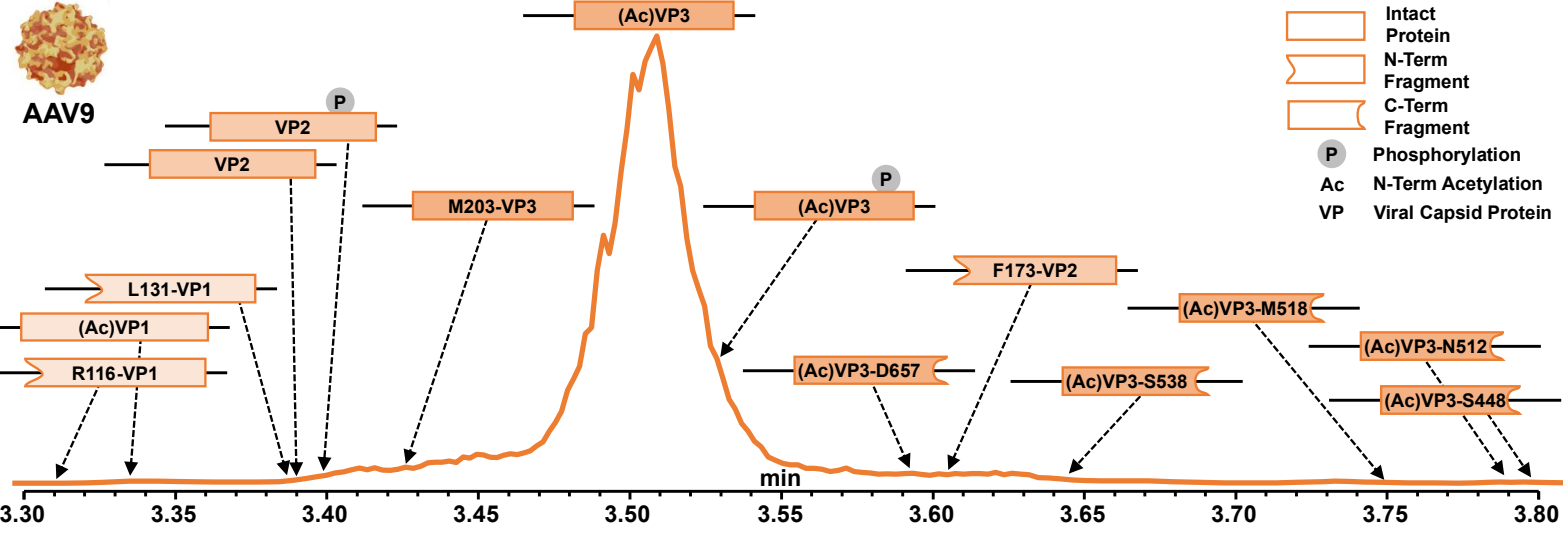
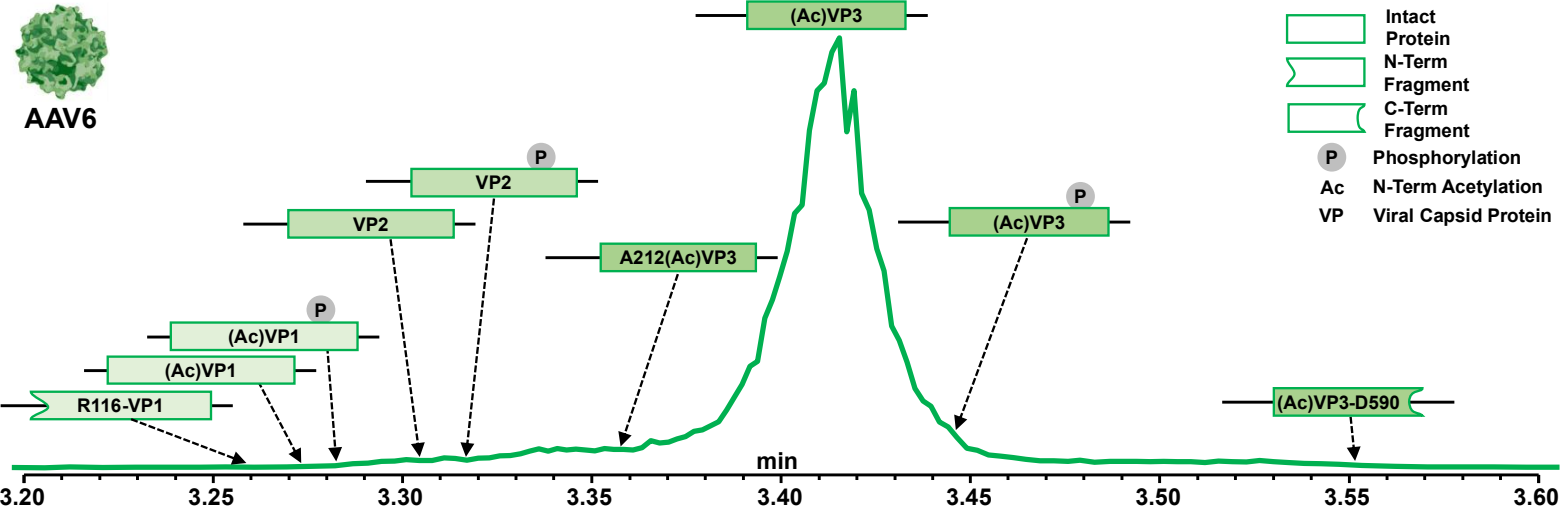
Broad Applicability of ZipChip Platform



Empty vs. Full Profiles



VP Proteoform Characterization



Adapted from Figure 3 of Smith et. al (Pre-print)

Adapted from Figure 4 of Smith et. al (Pre-print)

Detected VP Proteoforms and Fragments



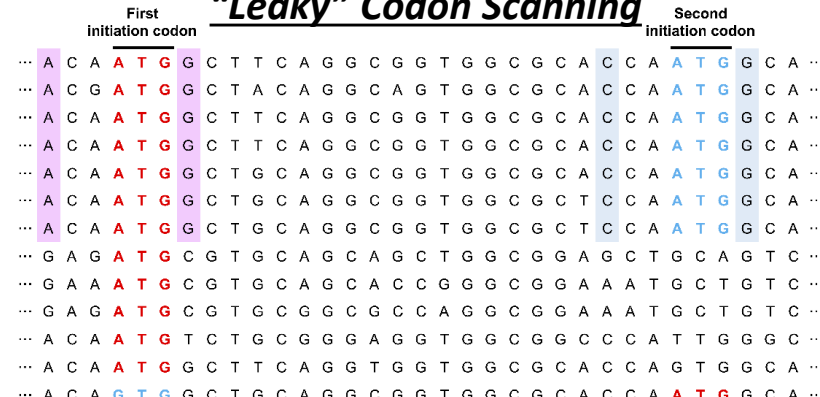
Start of AAV Sequence



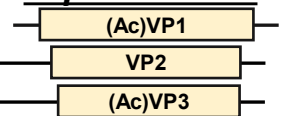
VP3 Variant Generation

Serotypes	N-terminal region		DP sequence		DG sequence		DP sequence	
	203	211	590 591	626 627	656 657			
AAV1	M A S G G G A P M A	M A	T D P A	T D G H	A N P P	...	...	...
AAV2	M A T G S G A P M A	M A	R Q A A	T D G H	A N P S	...	...	...
AAV3	M A S G G G A P M A	M A	T A P T	T D G H	A N P P	...	...	...
AAV6	M A S G G G A P M A	M A	T D P A	T D G H	A N P P	...	...	...
AAV8	M A A G G G A P M A	M A	T A P Q	T D G N	A D P P	...	...	...
AAV10	M A A G G G A P M A	M A	T G P I	T D G N	A D P P	...	...	...
AAVrh10	M A A G G G A P M A	M A	A A P I	T D G N	A D P P	...	...	...
AAV4	M R A A G G A A V	A V	N L P T	T D G H	A N P A	...	...	...
AAV11	M R A A P G G N A V	A V	T A P I	A D G H	A N P A	...	...	...
AAV12	M R A A P G G N A V	A V	T A P H	T D G H	A N P N	...	...	...
AAV5	M S A G G G P L G	A V	T A P A	T G A H	G N I -	...	...	...
AAV9	M A S G G G A P V A	A V	A Q A Q	T D G N	A D P P	...	...	...
AAV7	V A A G G G A P M A	M A	T A A Q	T D G N	A N P P	...	...	...

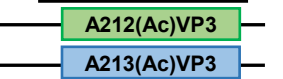
"Leaky" Codon Scanning



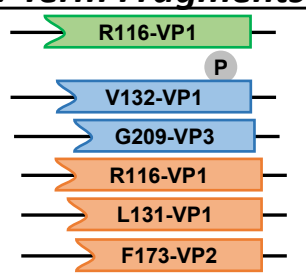
Expected VPs



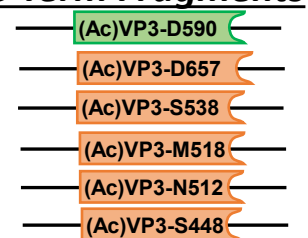
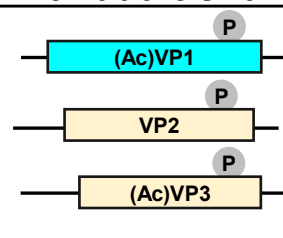
VP3 Variant



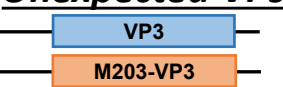
N-Term Fragments



VPs with additional PTMs C-Term Fragments



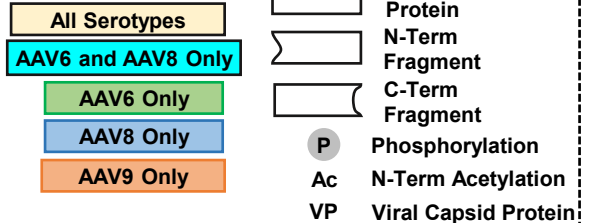
Unexpected VPs



Potential Causes of Fragments

- Baculoviral cathepsin
- Immune response
- Acidic conditions

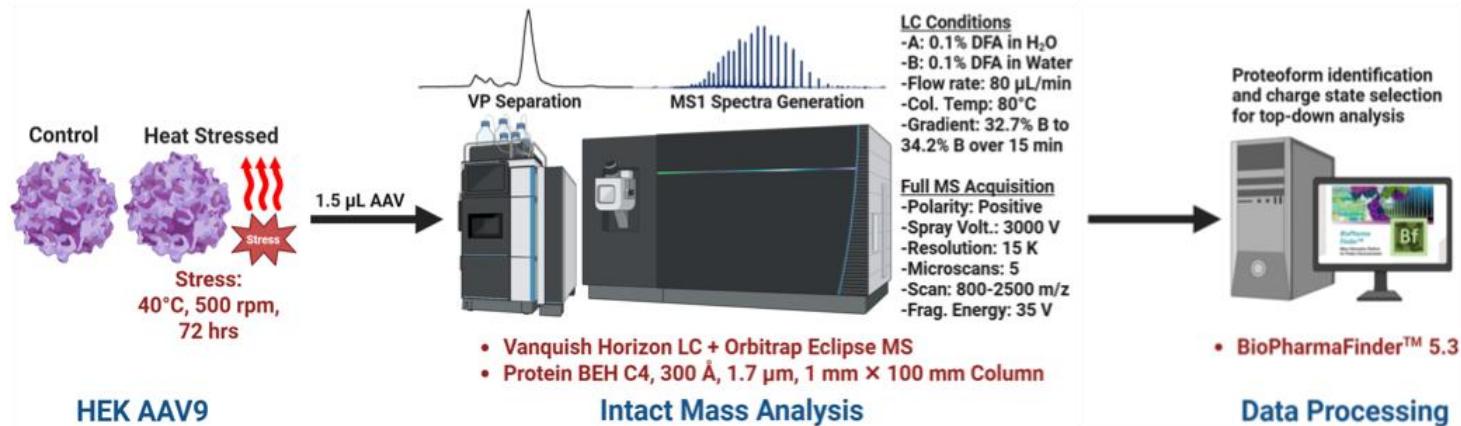
Key



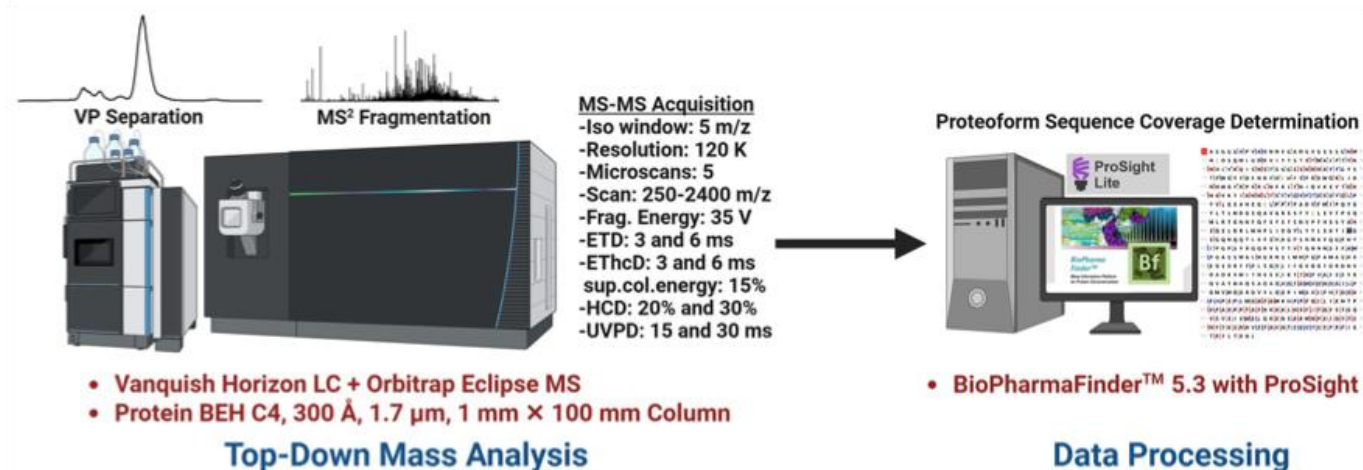
Adapted from Figure 5a of Oyama et al. (2021)
<https://www.liebertpub.com/doi/10.1089/hum.2021.009>

Adapted from Figure S4a of Oyama et al. (2021)
<https://www.liebertpub.com/doi/10.1089/hum.2021.009>

RP LC-MS/MS for Detection of Deamidation Events

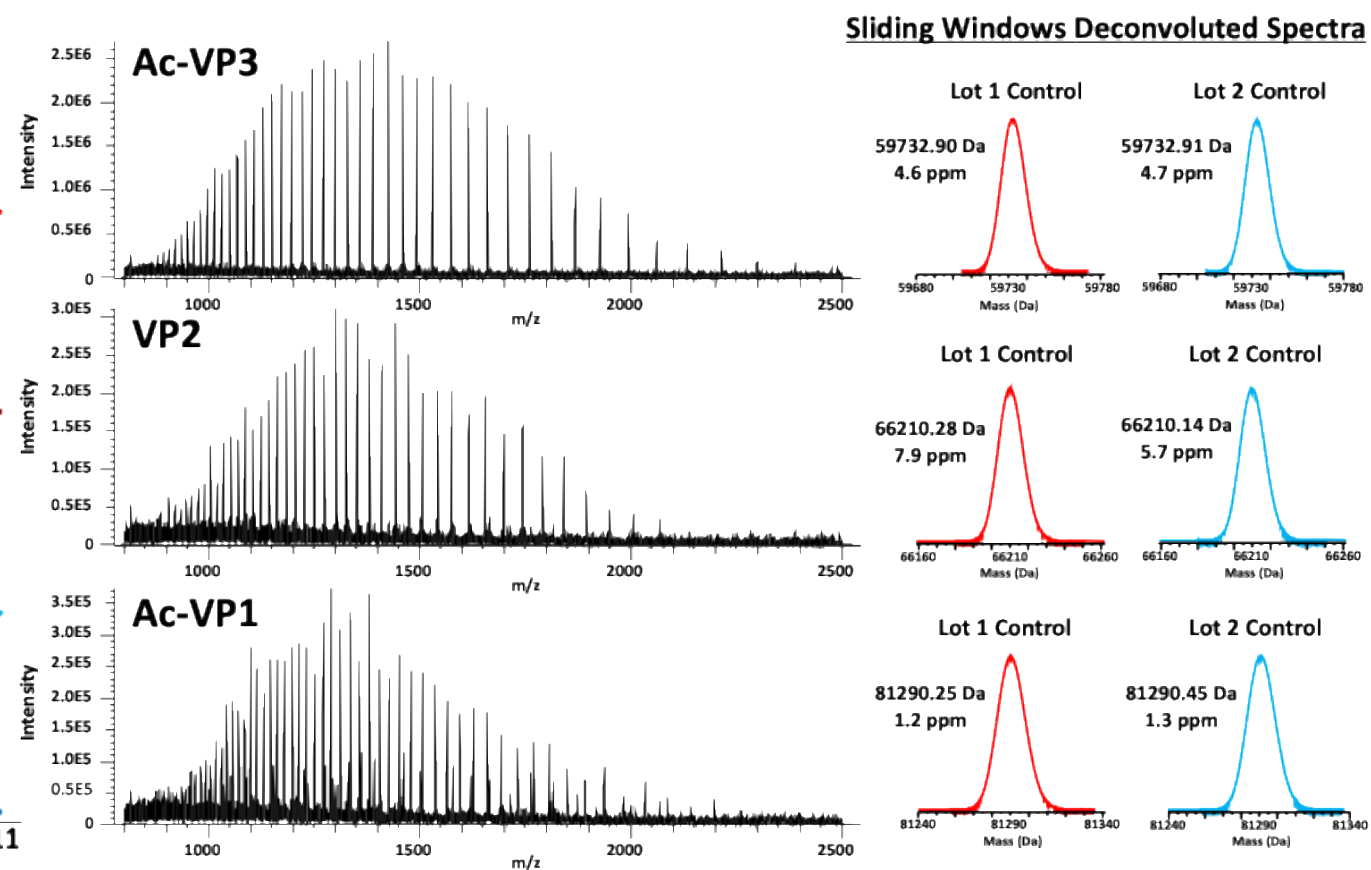
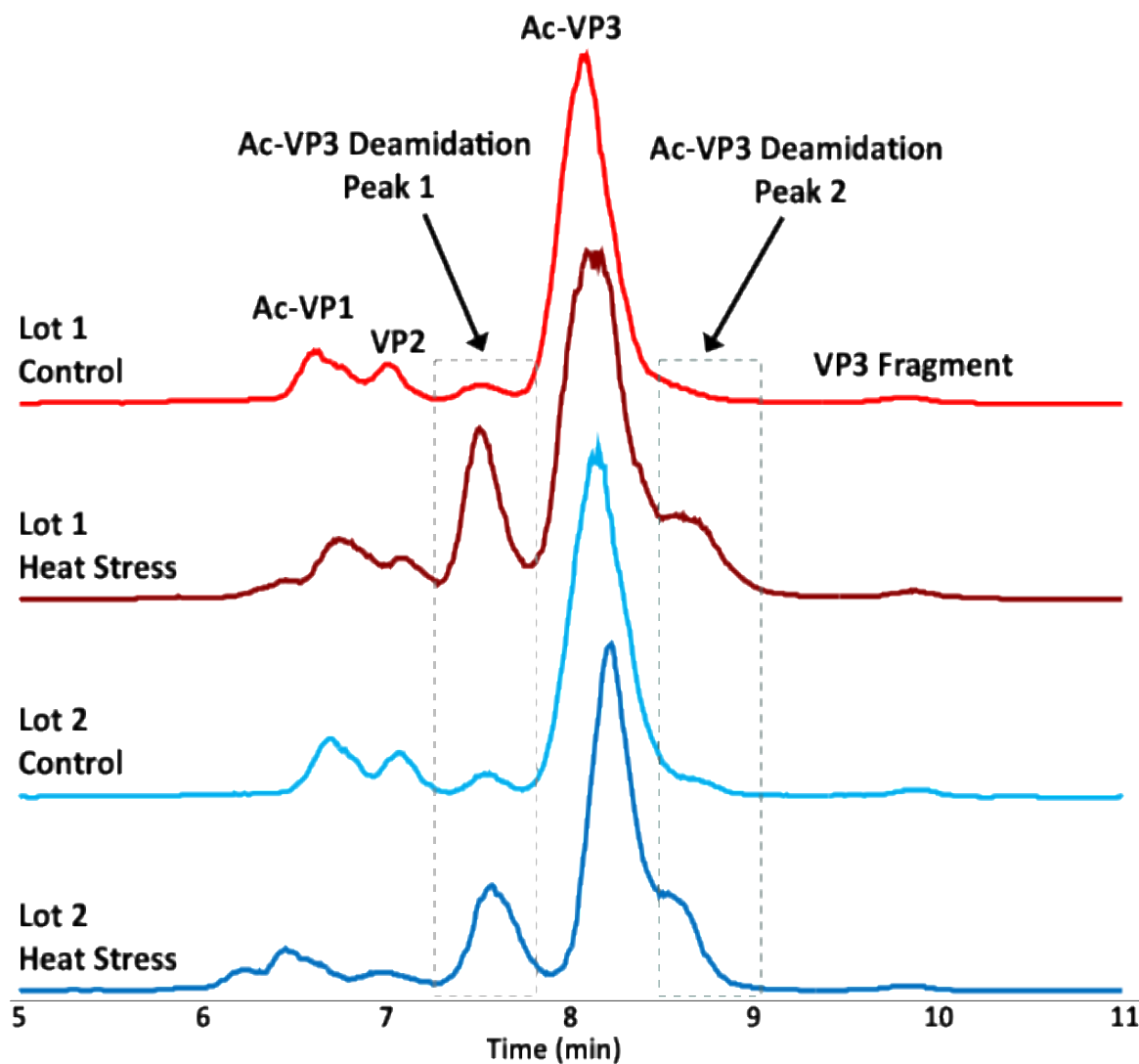


While the HILIC separation of VP's works well, for modifications such as deamidation events, HILIC does not have the necessary selectivity. Reversed-phase separation on C4 enables efficient separation of deamidated forms of the viral proteins.



RP LC-MS/MS for Detection of Deamidation Events

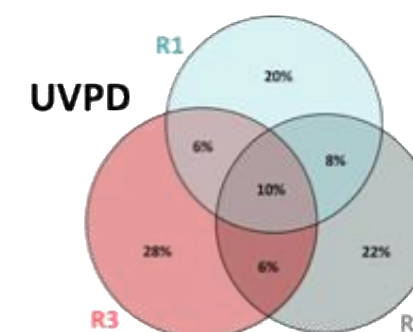
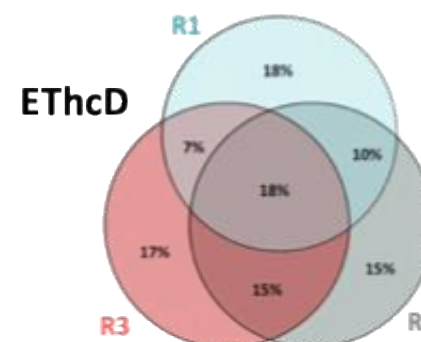
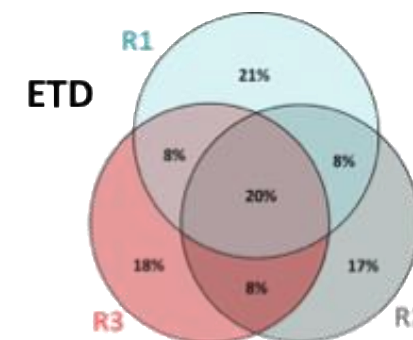
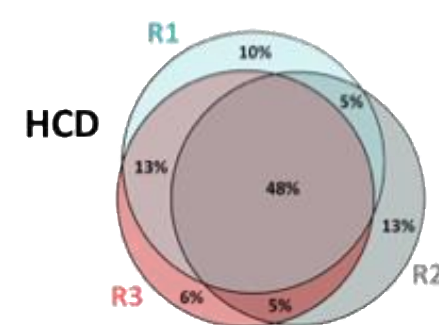
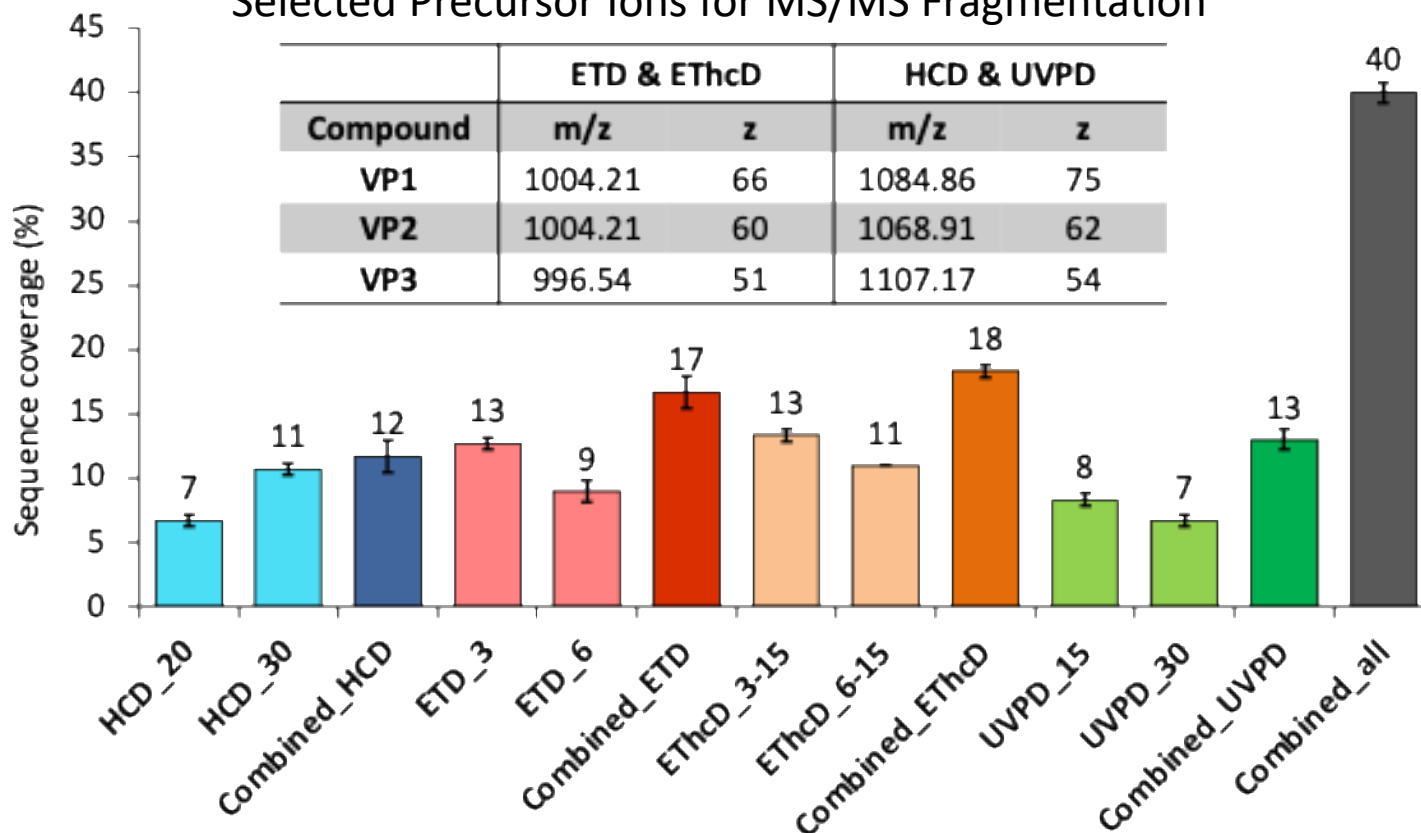
For deamidation events, HILIC does not have the necessary selectivity. C4 RP LC-MS enables efficient separation of deamidated forms of VPs.



Top-Down LC-MS/MS Using Different Ion Activation

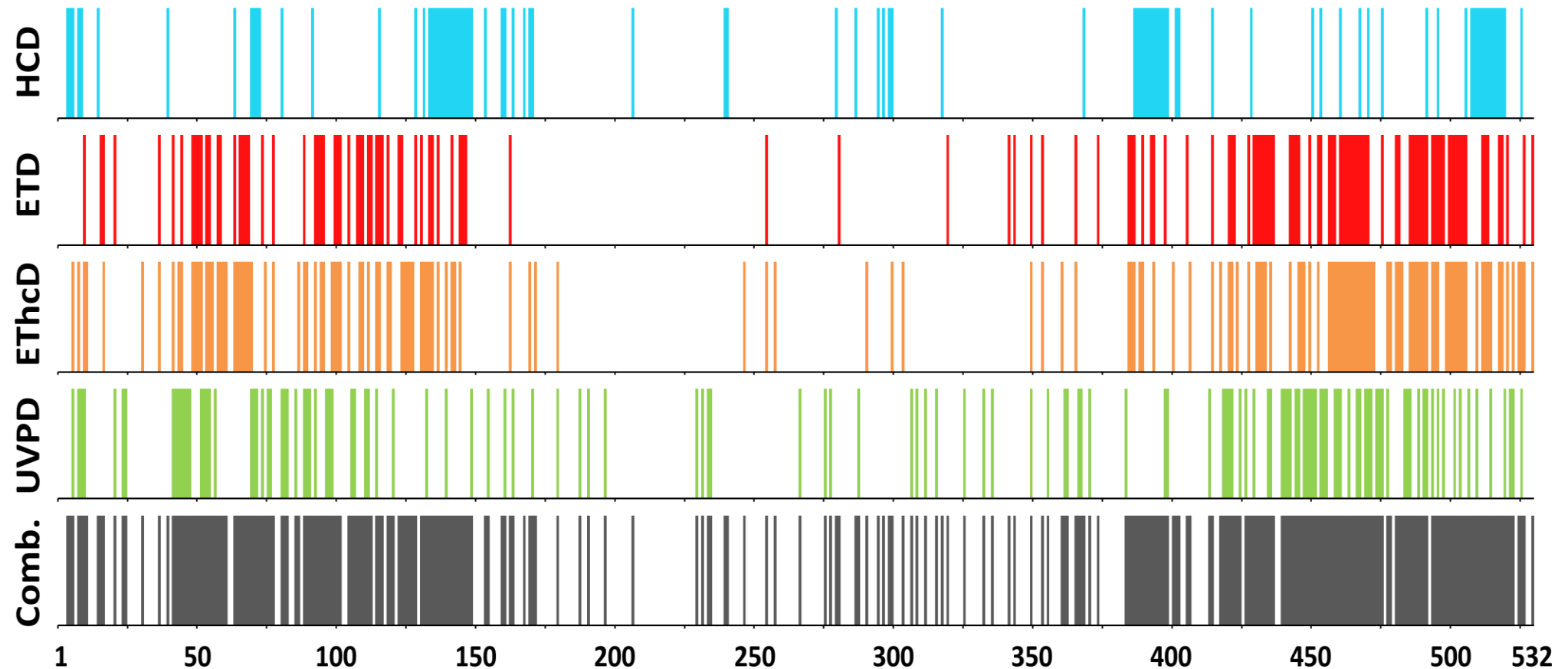
Selected Precursor Ions for MS/MS Fragmentation

Compound	ETD & EThcD		HCD & UVPD	
	m/z	z	m/z	z
VP1	1004.21	66	1084.86	75
VP2	1004.21	60	1068.91	62
VP3	996.54	51	1107.17	54



For deamidation events, HILIC does not have the necessary selectivity. C4 RP LC-MS enables efficient separation of deamidated forms of VPs.

Combined Top-Down MS/MS of VP3 on Orbitrap Eclipse

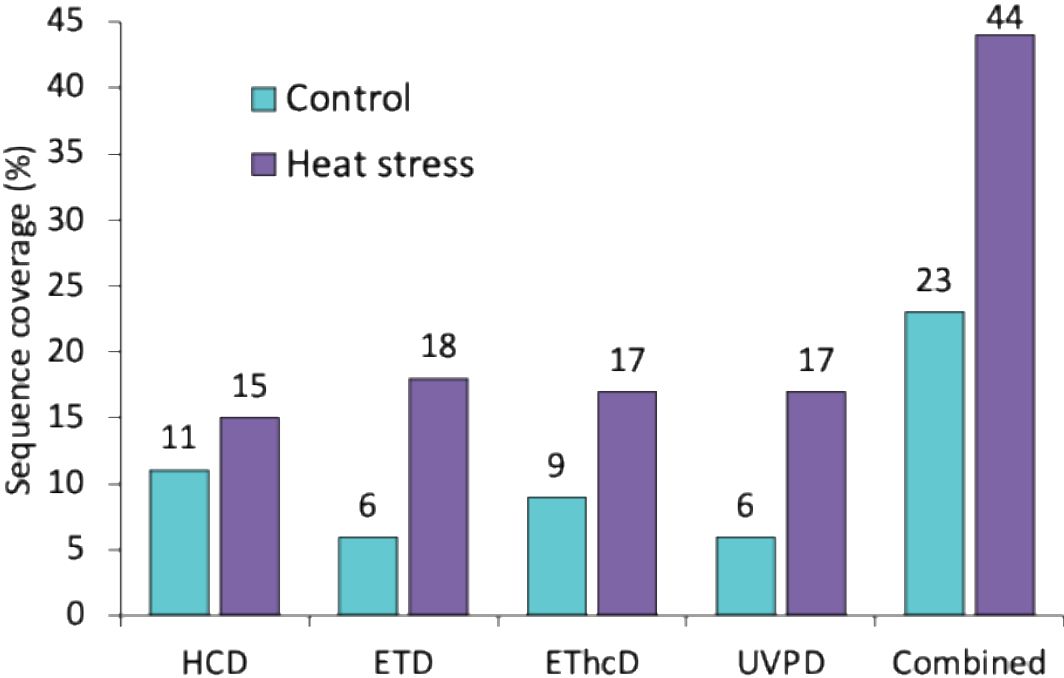


Bar code map depicting the fragmentation location in the amino acid sequence of the fragments detected in all fragmentation strategies using all replicates (n=12).

Top-down MS/MS Data Sequence Map of Deamidated VP3

Residue #	Peptides	Lot 1 Ctrl (%)	Lot 1 Heat Stress (%)	Lot 2 Ctrl (%)	Lot 2 Heat Stress (%)
N56+Deamidation (VP1 only)	YLGPNGNLDKGEPVNAADAAALEHDK (Y51-K76); YLGPNGNLDK (Y51-K60); YLGPNGNLDKGEPVNAADAAALEHDKAYDQQLK (Y51-K83)	14.28	41.80	15.87	47.60
N451+Deamidation (VP3:N249)	TINGSGQNQQTLK (T449-K461)	3.15	33.34	8.55	39.97

Peptide mapping identified N249 as a deamidation site. Top-down MS/MS of the Ac-VP3-DP1 peak also enabled identification of the deamidated residue. Overall performance of top-down LC-MS/MS is dependant on the abundance of the proteoform under study.



HCP Analysis Using Orbitrap Astral LC-MS



The Thermo Scientific™ Orbitrap™ Astral™ MS - Powered by the synergy of two synchronized HRAM analysers

ORBITRAP ANALYZER for high dynamic range HRAM MS and MS/MS

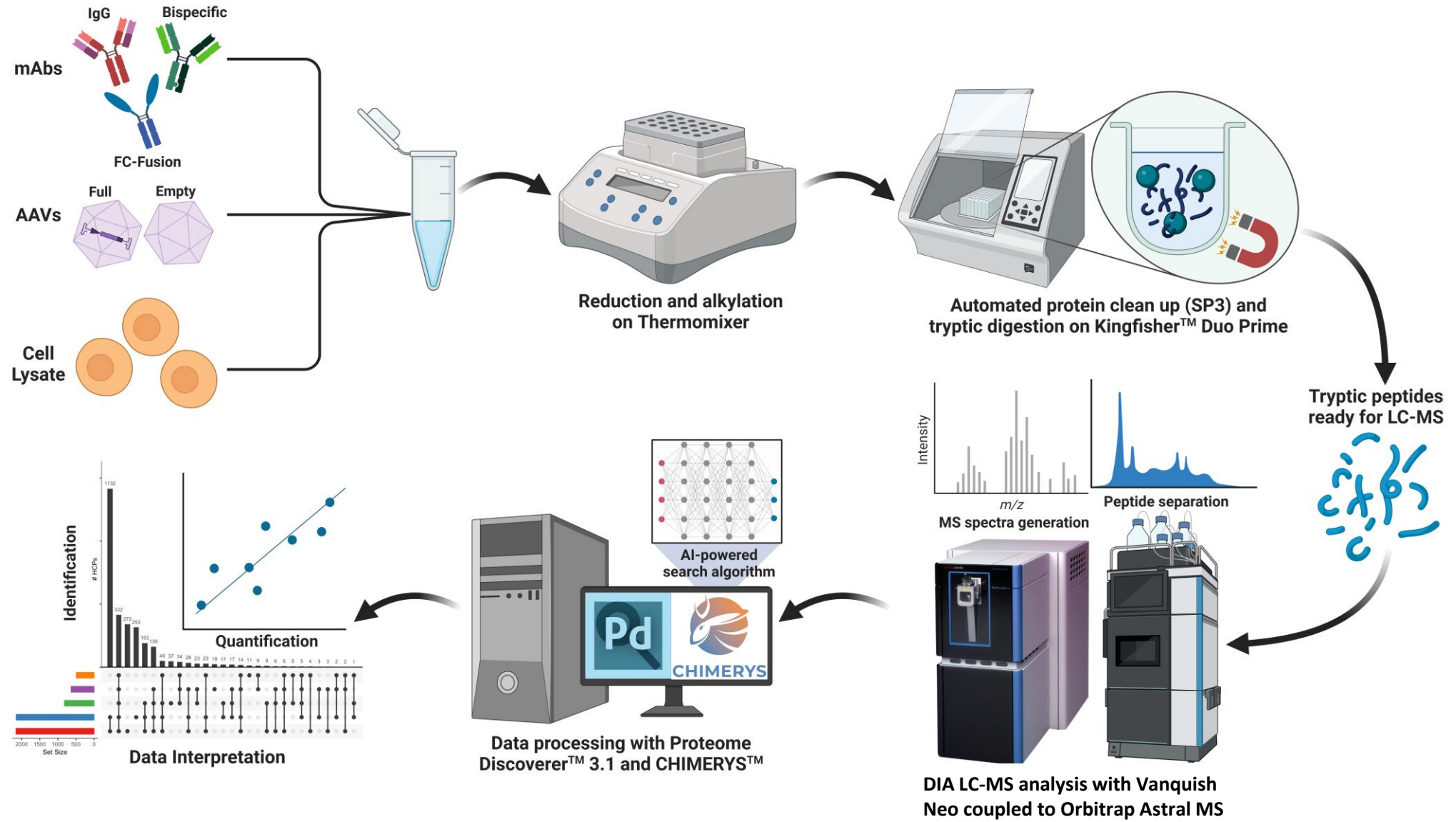
HRAM Scan Rate	Up to 40 Hz
Intrascan dynamic range	>5000 with single microscan
Max Resolution	480,000 at m/z 200
Mass Accuracy	RMS <3 ppm
Max m/z range	Up to m/z 8000 with Biopharma Option



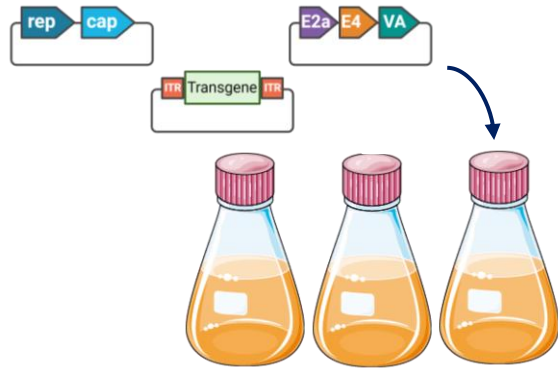
ASTRAL ANALYZER for fast and sensitive high dynamic range HRAM SIM and MS/MS

Sensitivity	Single ion detection
HRAM Scan Rate	Up to 200 Hz
Intrascan dynamic range	>1000 with single microscan
Resolution	80,000 at m/z 524
Mass Accuracy	RMS <5 ppm

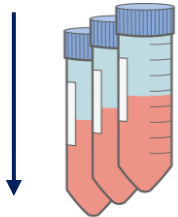
Sample Preparation Workflow



Tracking HCP Clearance using AAVX Affinity Chromatography

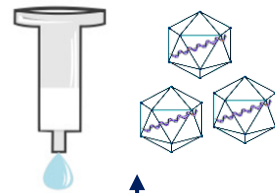


AAV produced in HEK cells using GIBCO AAV-MAX platform

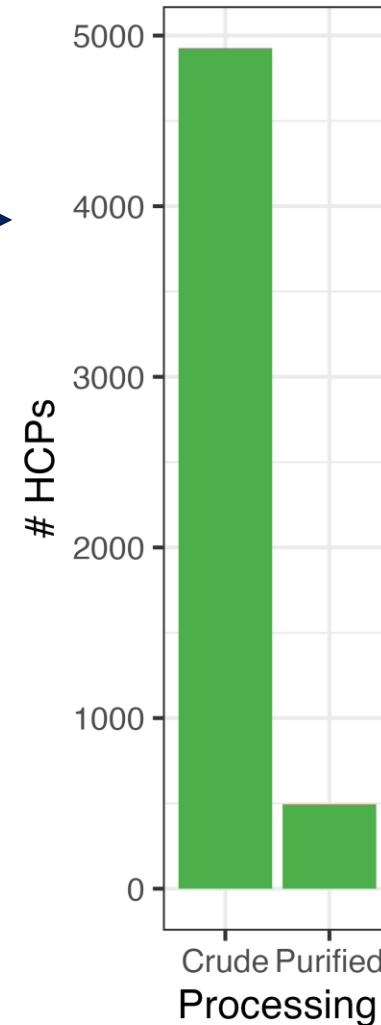


Conditioned media collected, sample retained, remainder purified using POROS CaptureSelect™ AAVX Affinity Resin pre-packed column

Purified AAV buffer exchanged to PBS containing 0.01% Pluronic using centrifugal filters



FPLC

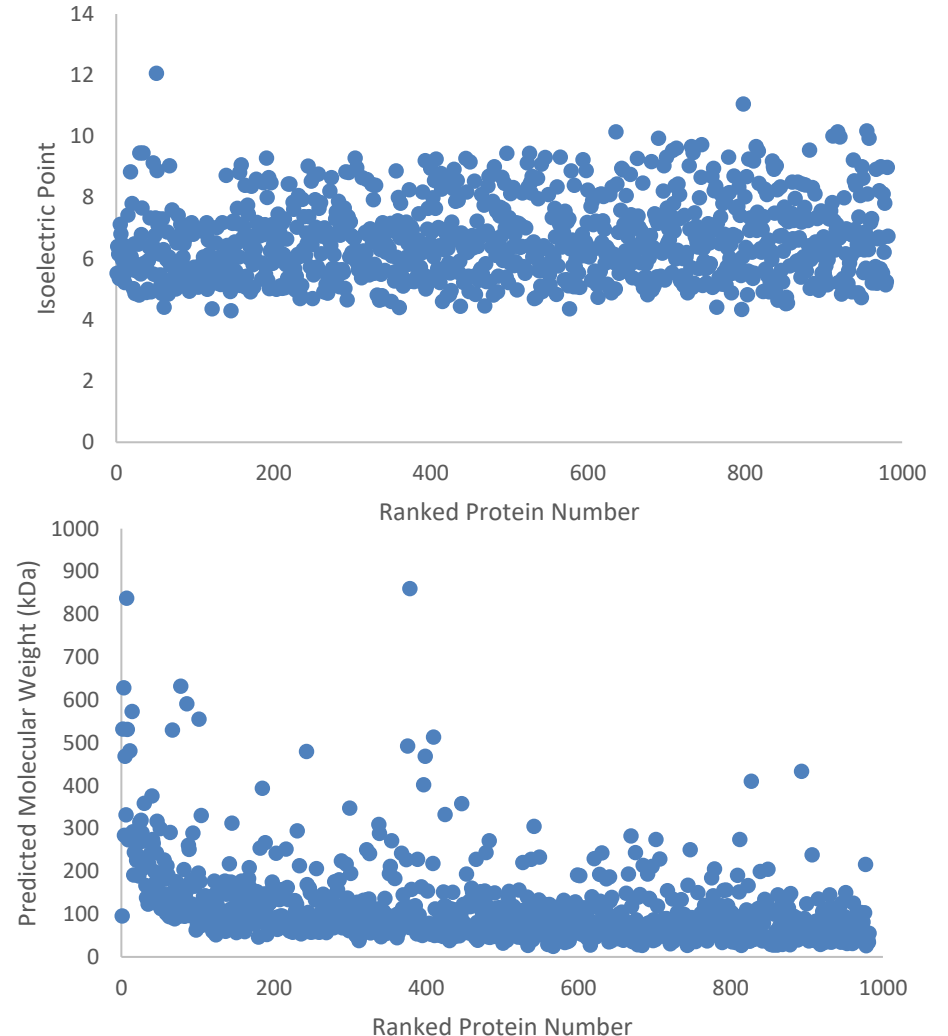


Purification

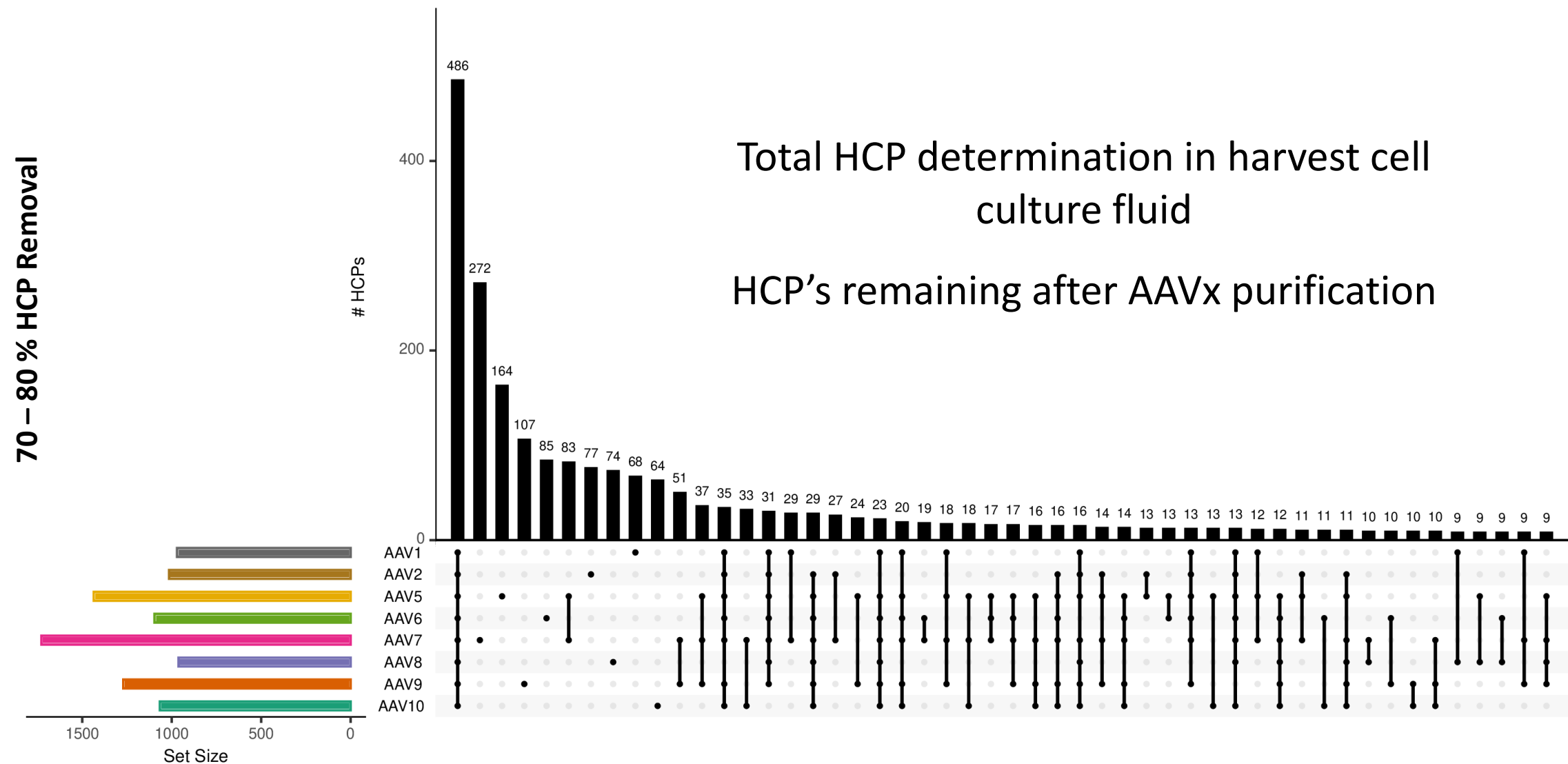
- AAV and AAV Associated Proteins
- Adenovirus C Serotype 5 Proteins
- Human Proteins

HCPs Associated with Purified CMV-GFP AAV8

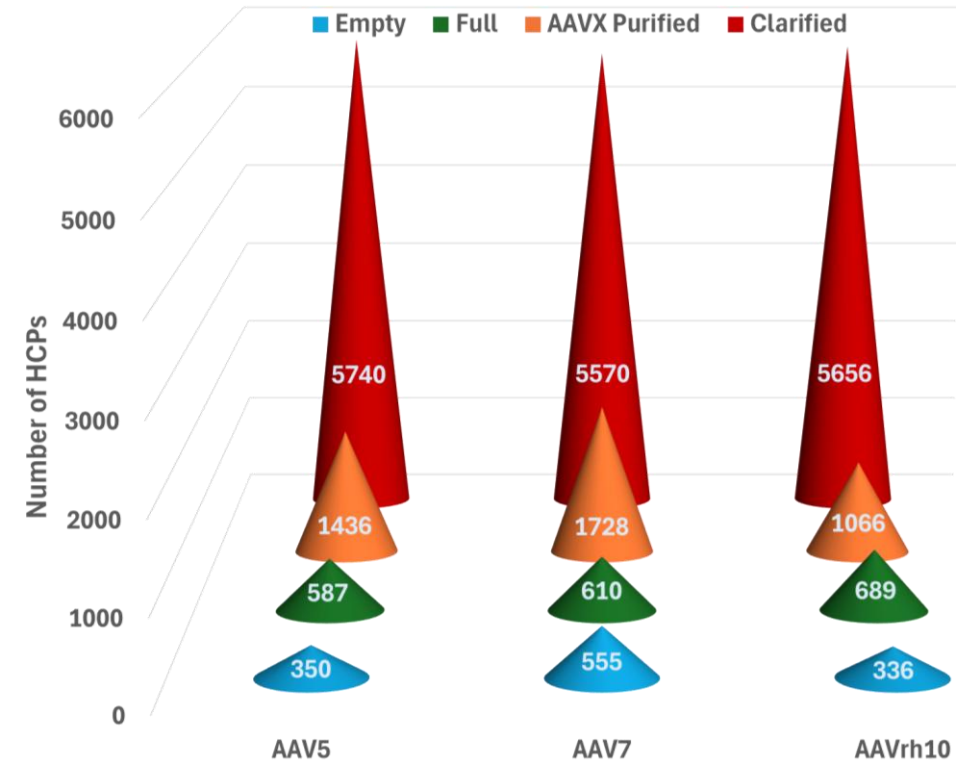
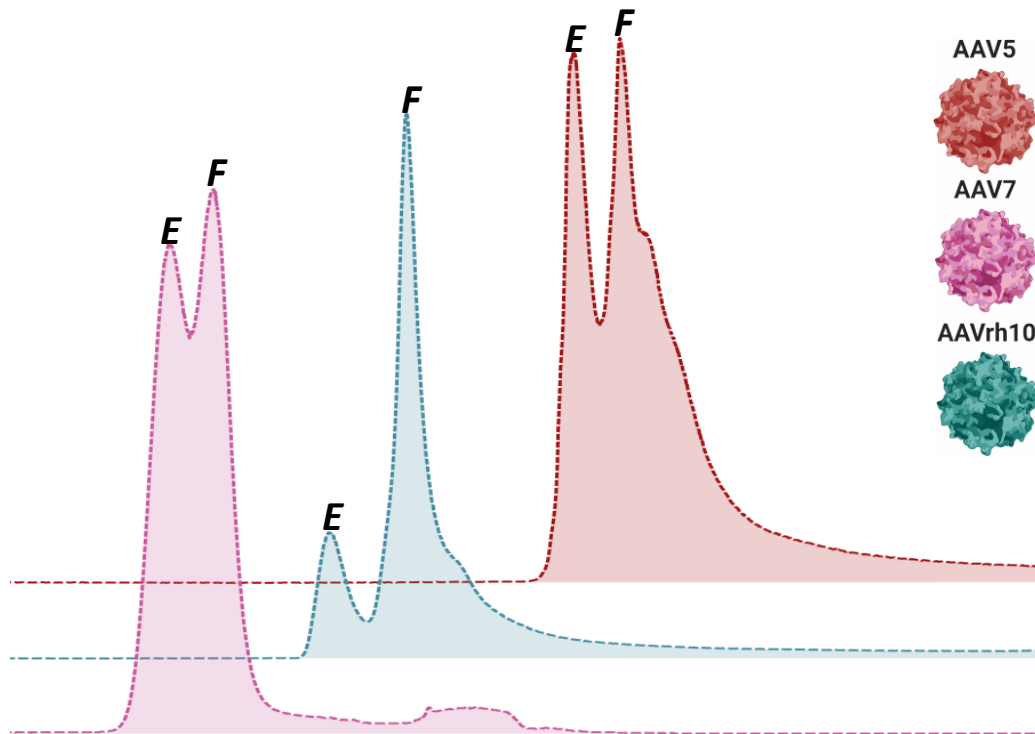
- ❑ AAVX purification resulted in ~80% reduction in the levels of HCPs present in the process stream using a simple bind and elute method.
- ❑ For proteins associated with the retained viral capsids, GO terms relating to binding, in particular protein binding (92.7% of the total set) were enriched. 97.1% were mapped as being intracellular proteins.
- ❑ Standard physiochemical parameters were explored including molecular mass, pI, hydrophobicity *etc.* However, distributions were broad and as expected, no correlation existed.



Exploring HCP Distribution Across Various AAV Serotypes

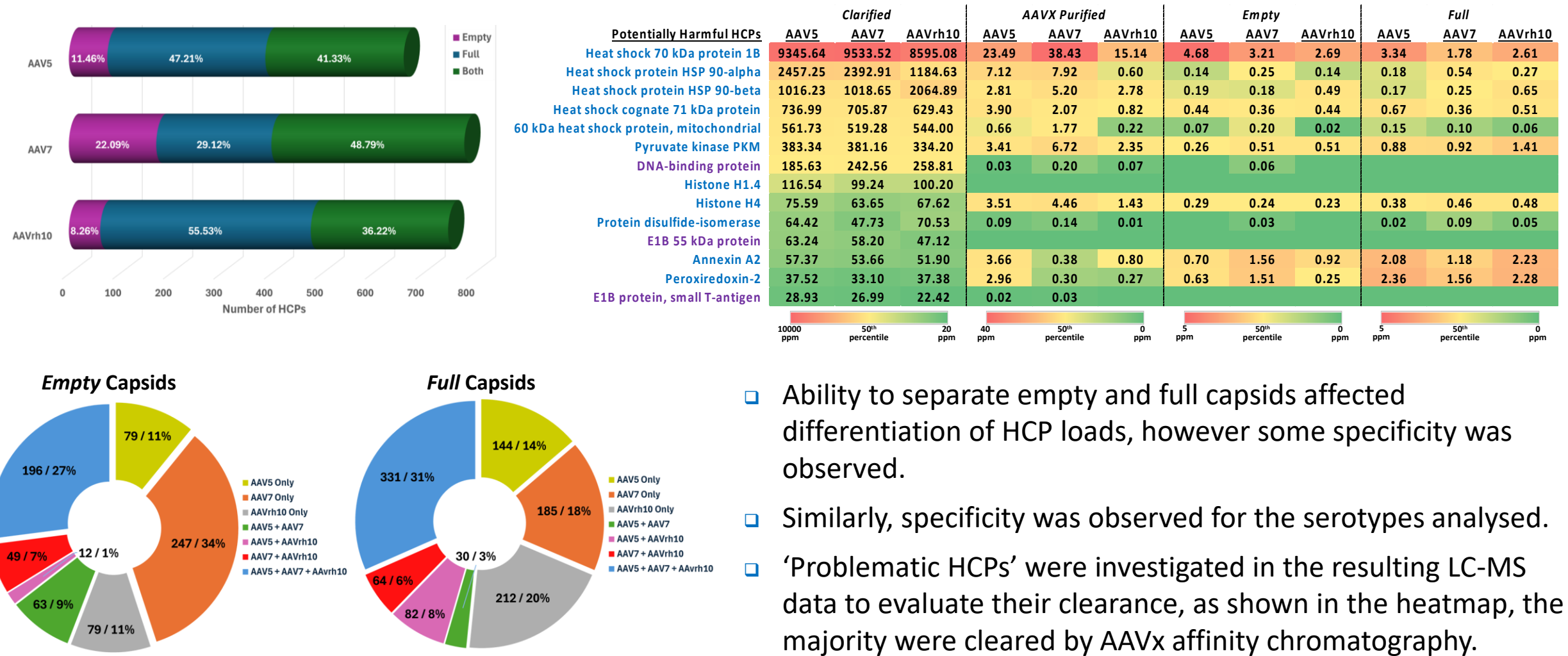


Monitoring Clearance Using Two-Step Downstream Processing



Post AAVX affinity purification, anion exchange separation of empty and full capsids were performed using Poros XQ. Fractions were collected and analysed by LC-MS on Orbitrap Astral to investigate clearance of the HCPs and distribution across the different capsid fill states.

Distribution of HCPs across Empty and Full Capsids



Summary

- ❑ Native MS and CDMS can be coupled with upfront anion exchange chromatography for confirmation of capsid fill state. Partial capsids not observed either by chromatography or MS, thought to be due to GOI size.
- ❑ Viral protein separation possible using various chemistries, HILIC method works well and is simple to deploy, however, reversed-phase outperforms for separation of deamidated forms.
- ❑ Top-down MS/MS showing strong potential for VP specific characterisation. Combination of different ion activation strategies on tribrid MS instrument enabled excellent N- and C-terminal fragmentation.
- ❑ HCP behaviour investigated using throughout the downstream process for HEK293 derived serotypes using Orbitrap Astral. Some specificity identified based on the serotype and capsid fill state, however, AAVx affinity chromatography enables bulk clearance.

Acknowledgements

NIBRT:

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908 Devices:

Erin Redman