

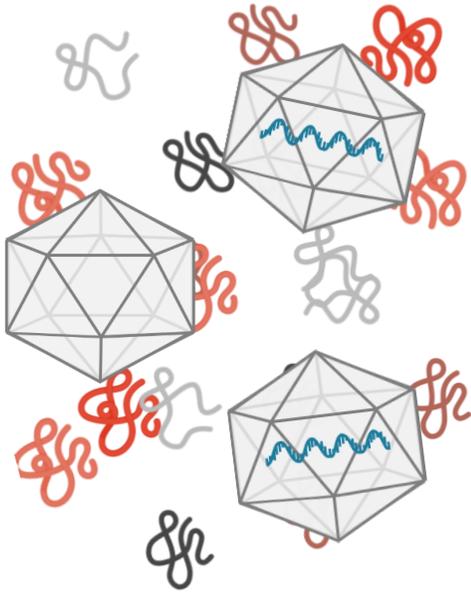
Characterising Viral Vectors for Gene Therapy using Mass Spectrometry on Different Levels

Josh Smith¹, Corentin Beaumal¹, Felipe Guapo¹, Lisa Strasser¹, Florian Füssl¹, Silvia Millán-Martín¹, Sara Carillo¹, Aaron Richardson¹, Colin Clarke^{1,2} & Jonathan Bones^{1,2}

¹NIBRT, Foster Avenue, Mount Merrion, Blackrock, Co. Dublin, A94 X099, Ireland.

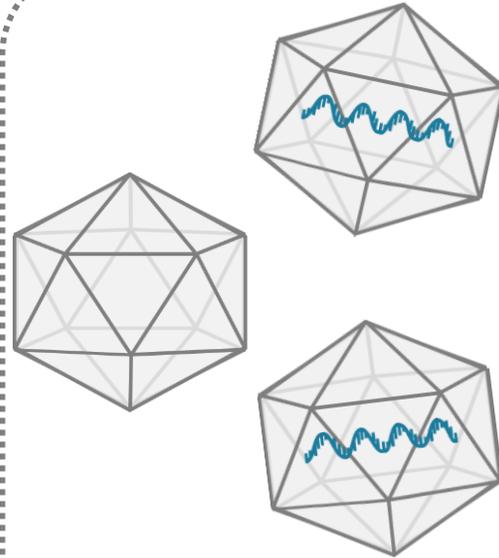
²School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, D04 V1W8, Ireland.

Characterisation of AAV using Multiple Levels of Analysis



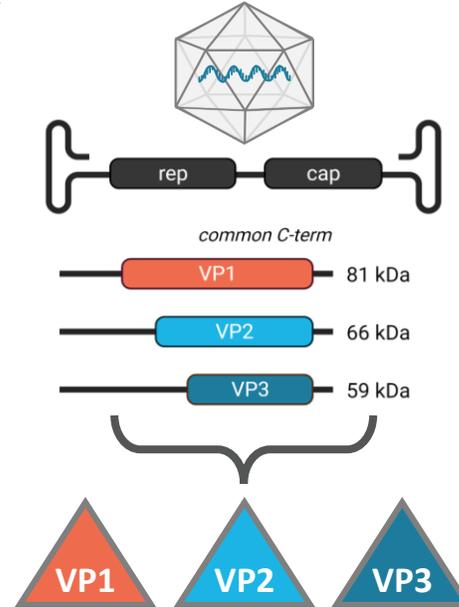
Host cell proteins:

Determine and quantify residual process contaminants



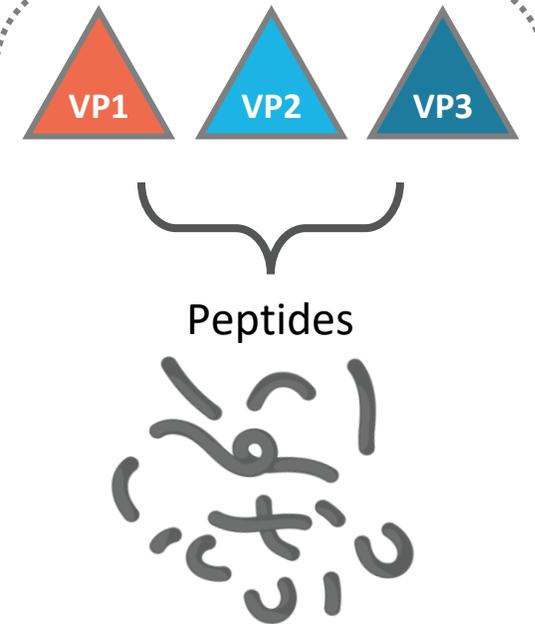
Intact capsids:

E:F ratio assessment
Determine fill state



Intact capsid proteins:

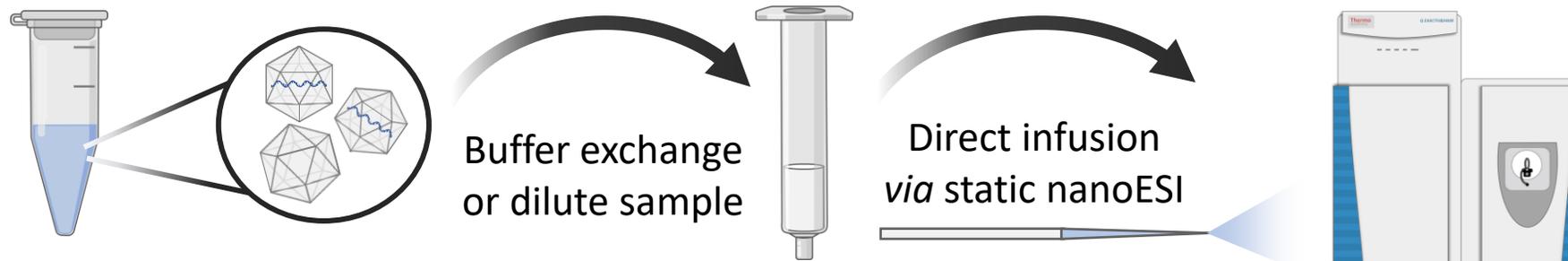
Determination of VP ratio → capsid stoichiometry



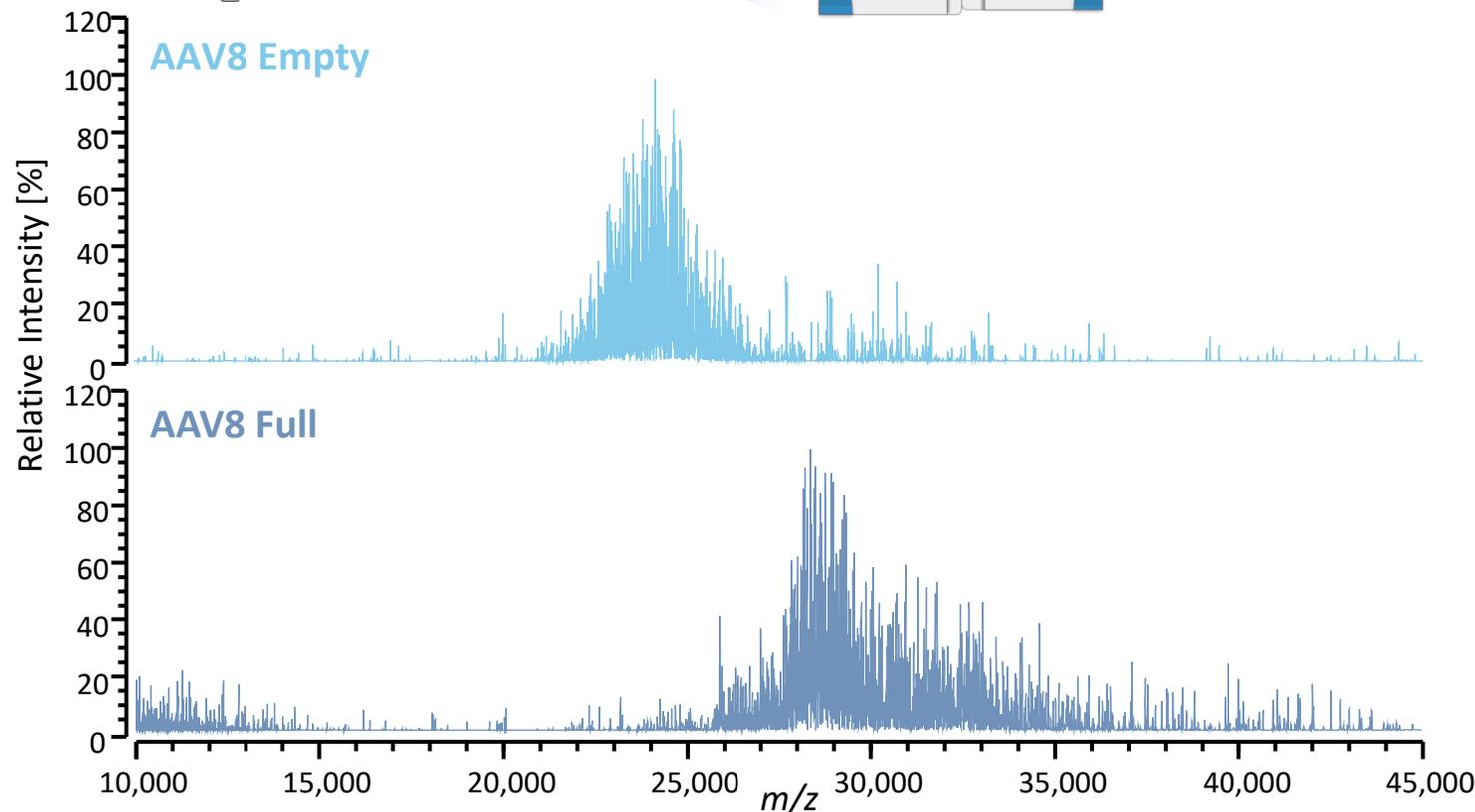
Peptide level:

Determination of AA sequence and PTM detection

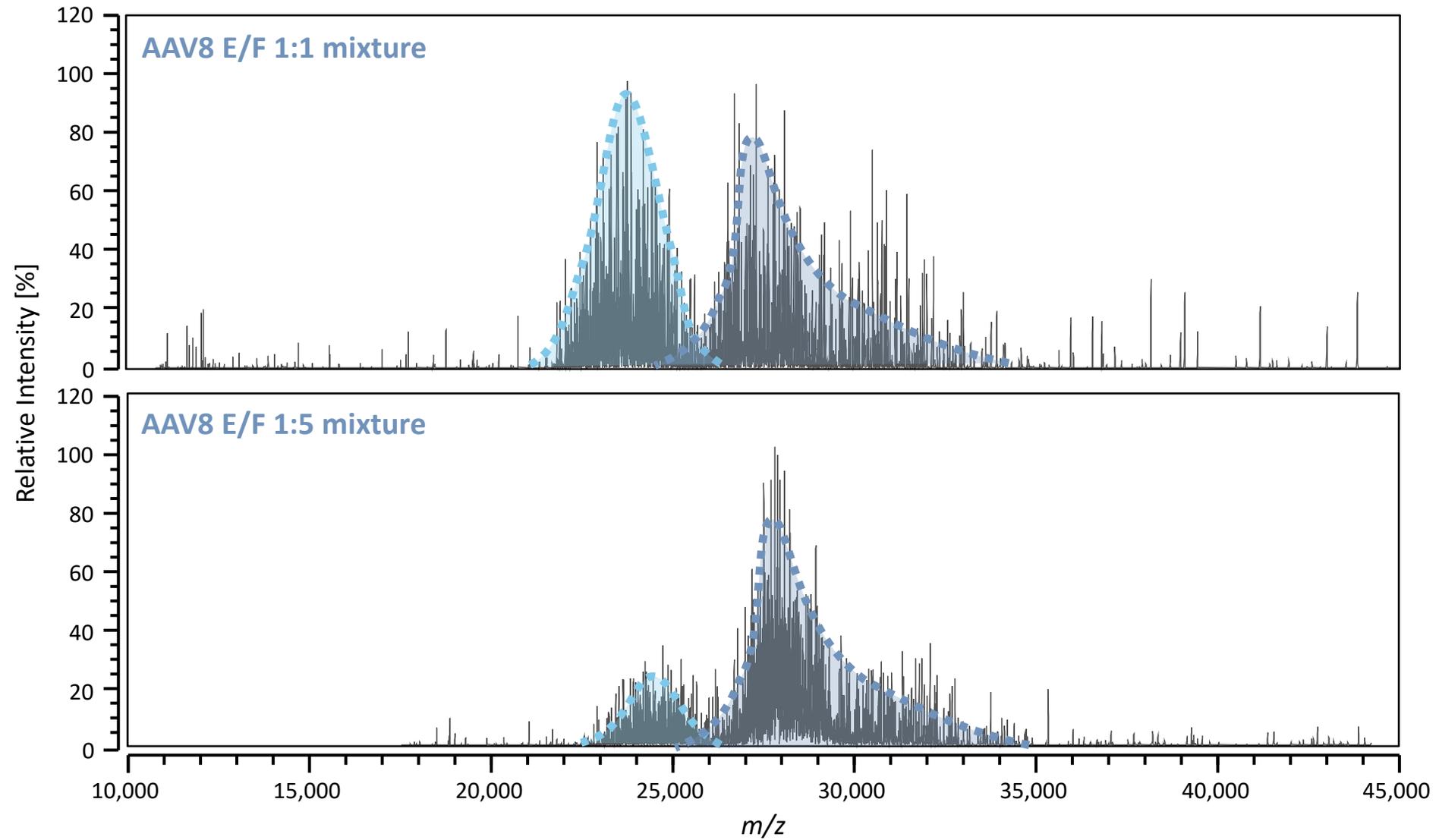
Capsid Fill State Assessment Using Native MS



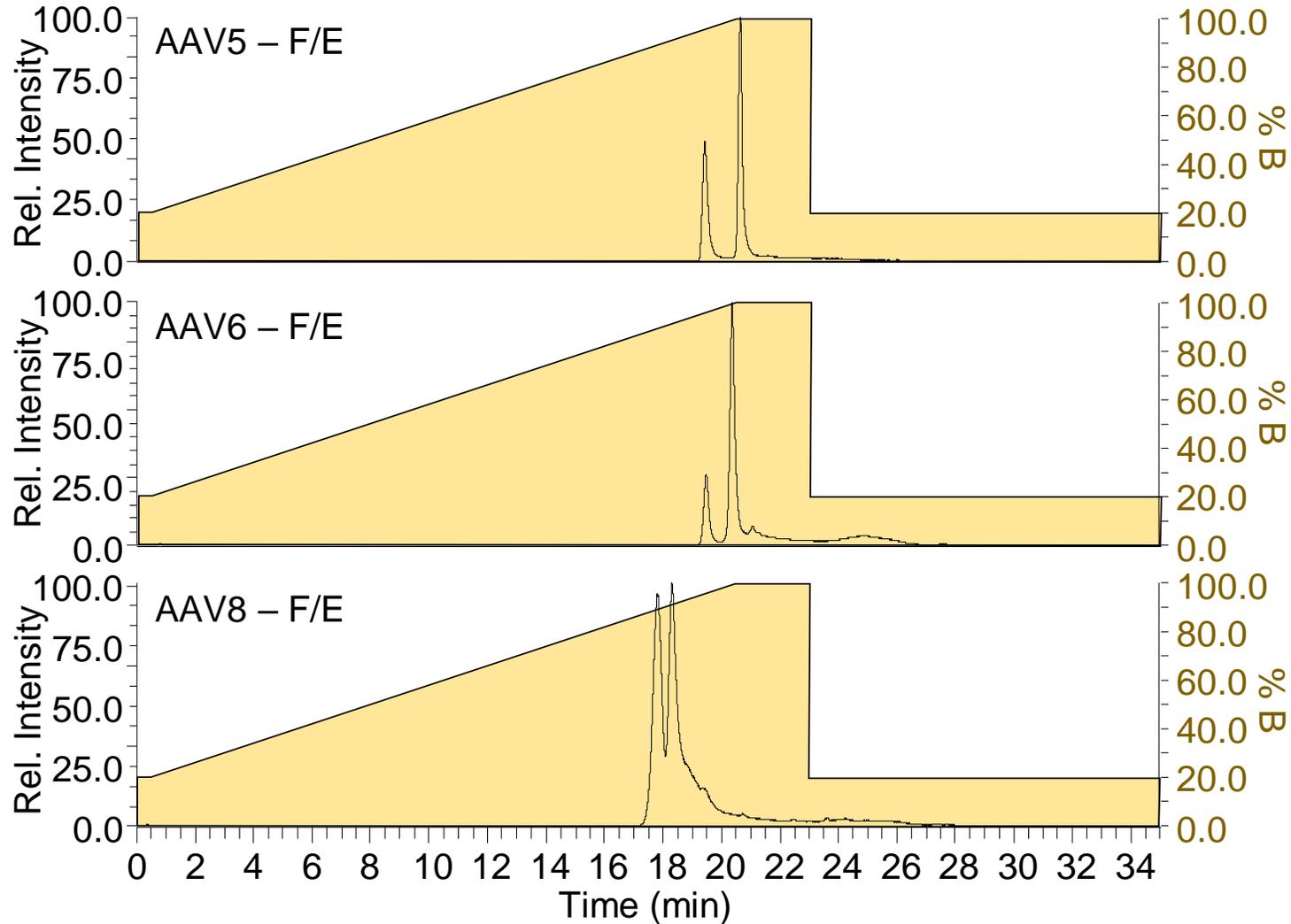
Thermo Q Exactive UHMR	Setting
Resolution	25,000 at m/z 200
Microscans	10
AGC Target	1e06
Max. IT	200 ms
In-source trapping	-100 V
Source DC offset	-50 V
Extended trapping	150 V
Trapping gas	SF ₆ at 4e-10 mbar
Acquisition	5 mins with transient averaging enabled



Cluster Areas Correlate With Abundance of Capsid Species

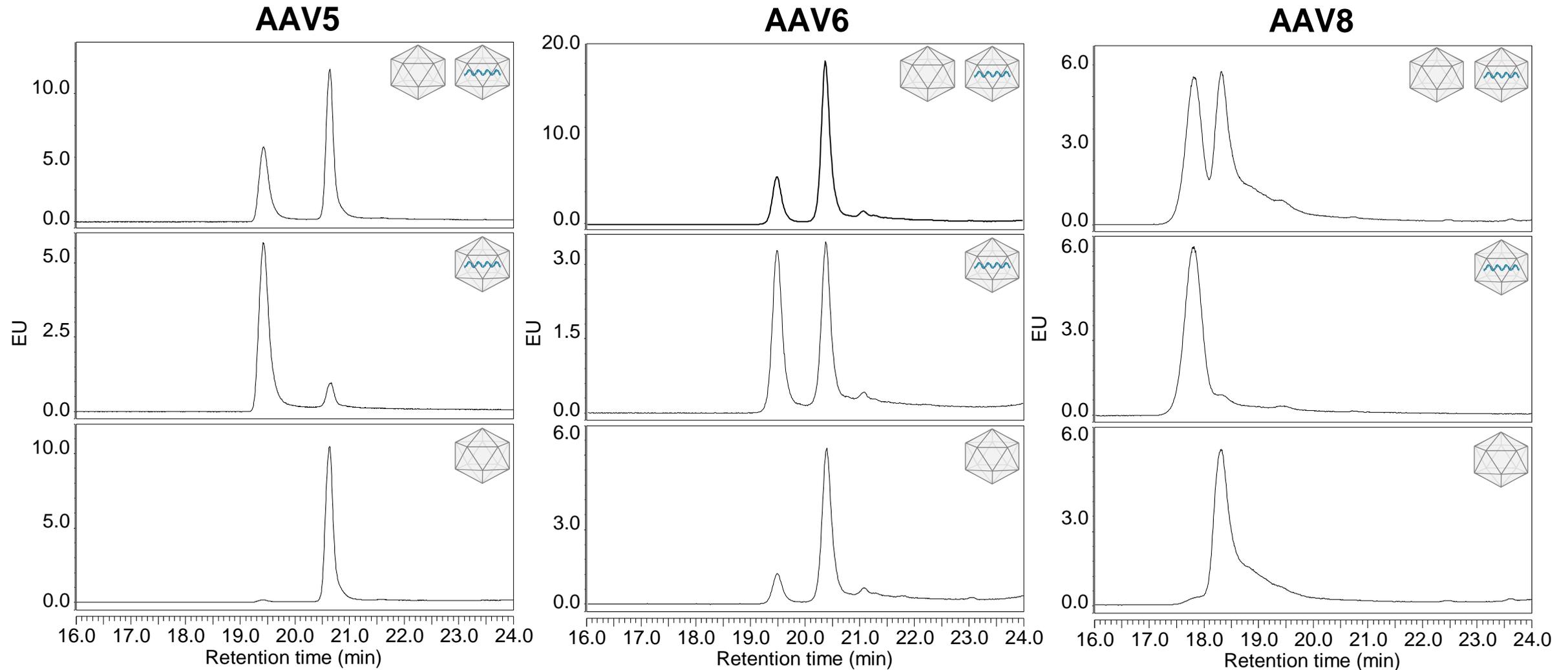


Coupling with Anion Exchange Chromatography

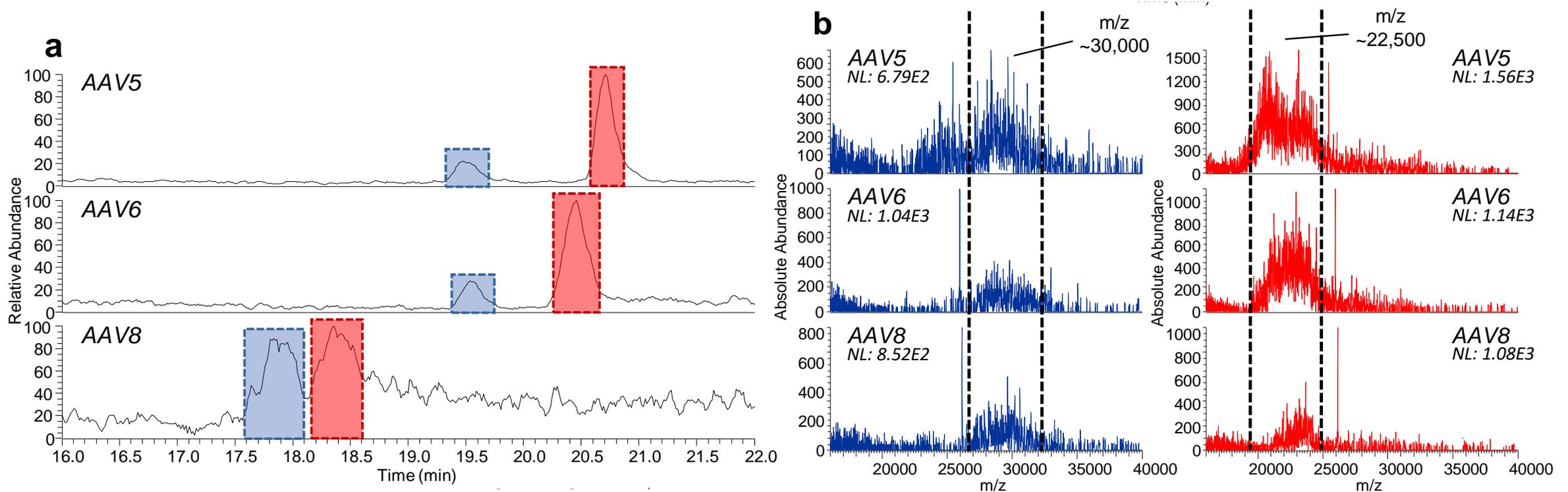


- pH gradient anion exchange separation of full and empty capsids using Thermo Scientific ProPac 3R AEX column.
- Gradient specifically designed to be generic for different serotypes and mass spectrometry compatible.
- pH gradients enable focusing effect, elution occurs when gradient pH = analyte pI,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

Determination of Capsid Fill State Elution Order

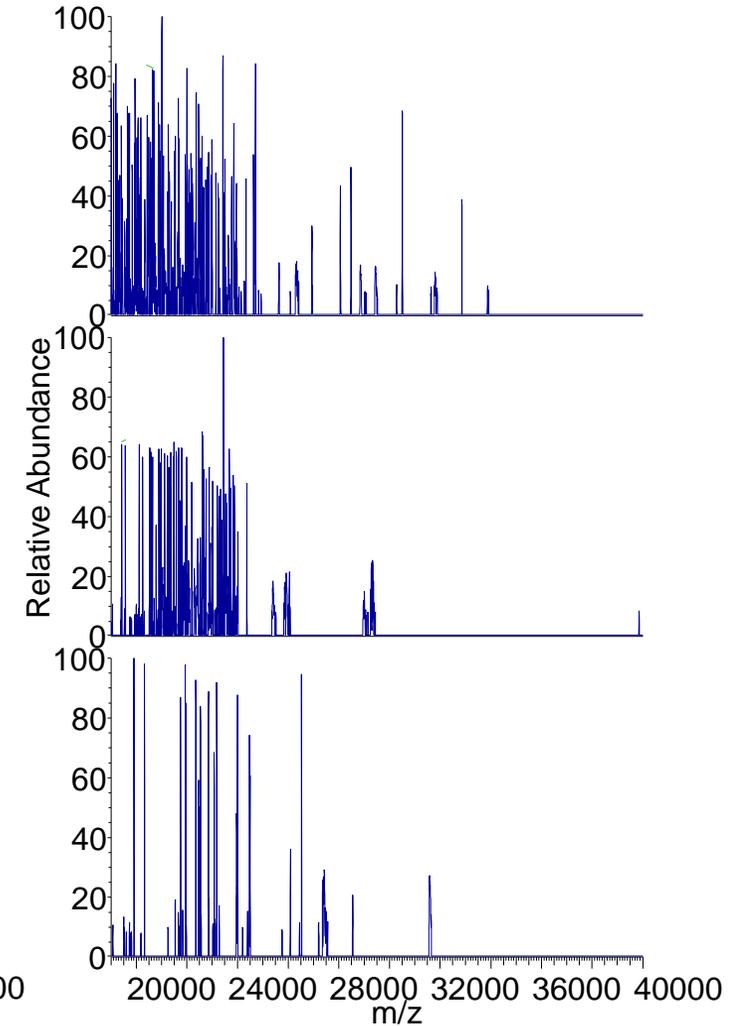
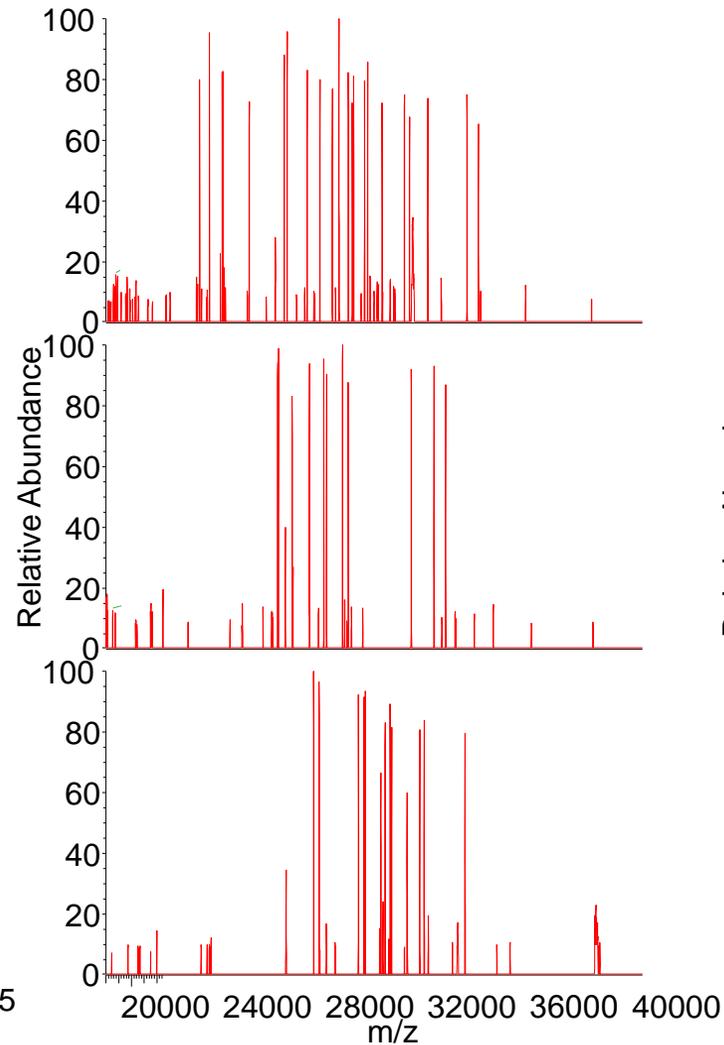
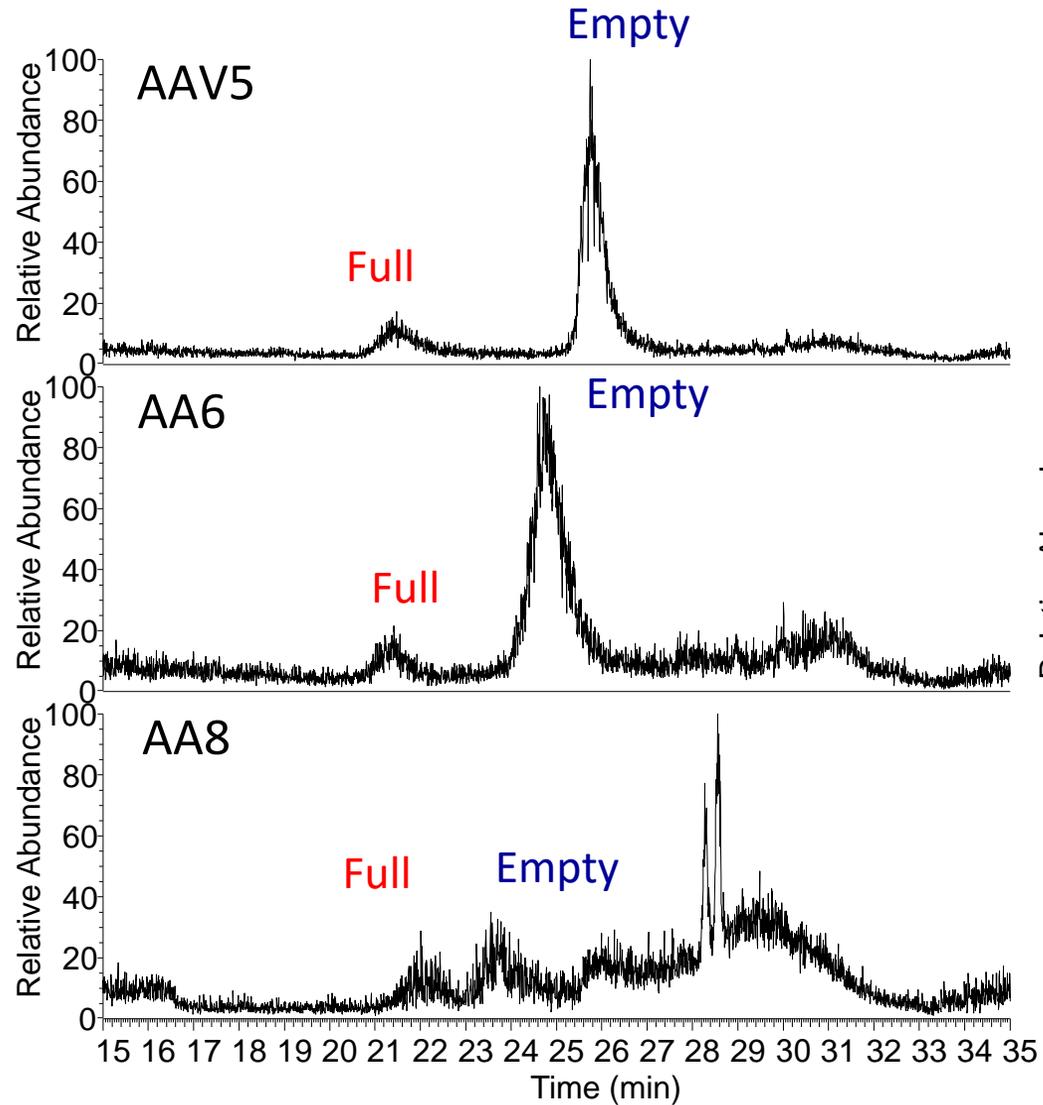


Coupling with Native MS Detection

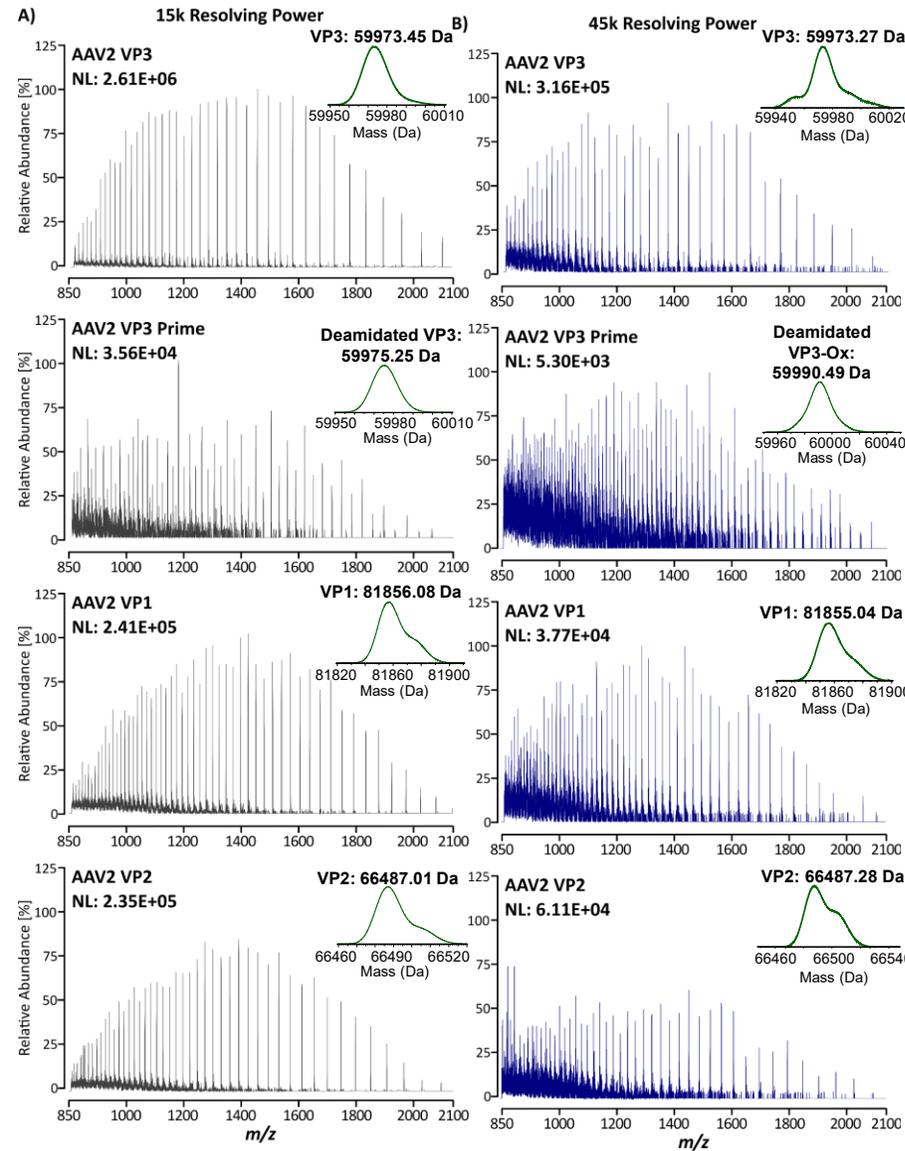
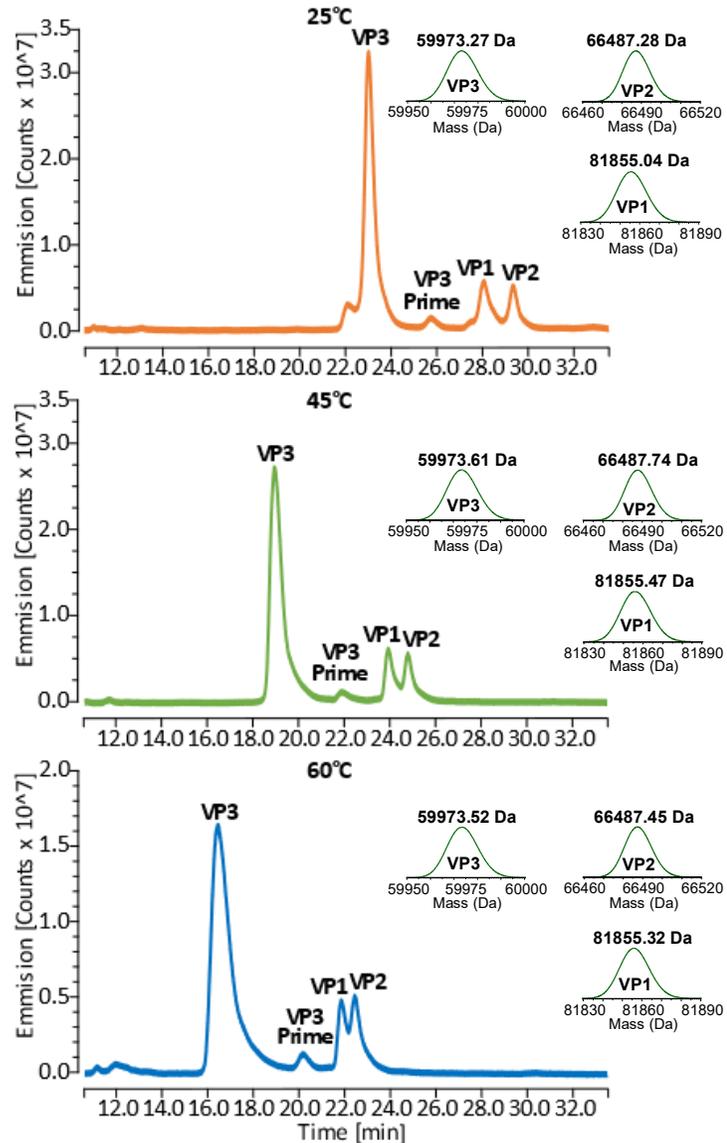


- pH gradient anion exchanged coupled directly to Thermo Scientific Q Exactive UHMR mass spectrometer for confirmation of capsid fill state species identification based on m/z.
- Assuming similar charge, earlier eluting peak contains heavier species explained by the presence of cargo DNA, additional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

Coupling CDMS with Front End F/E AEX Separation



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



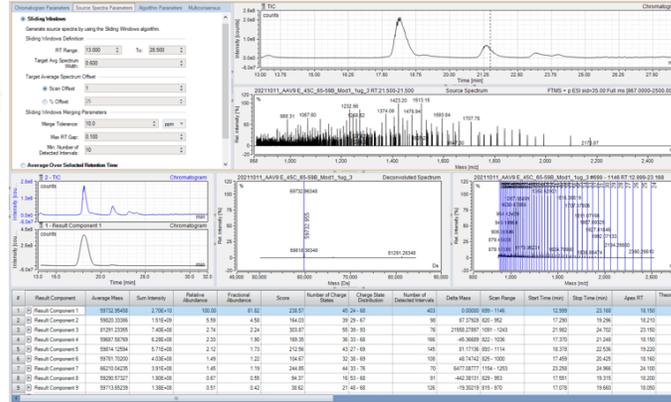
BioPharma Finder Software 4.1



Chromleon 7.3 CDS No compromise

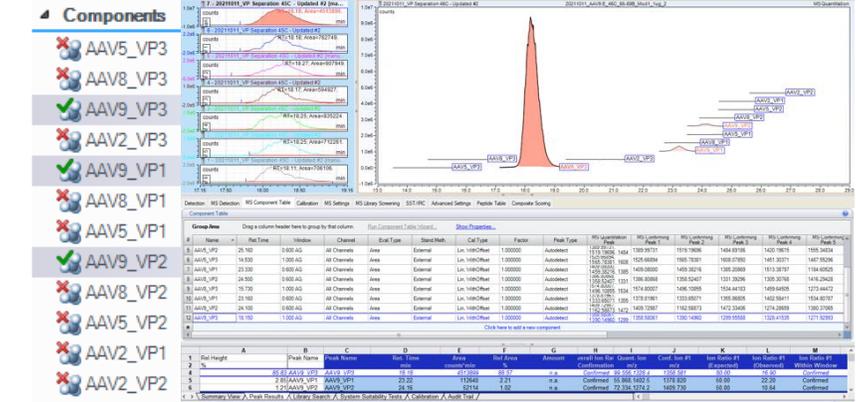
Processing Method and Component List Importation from BPF

Intact Protein Deconvolution



Serotype Profiling

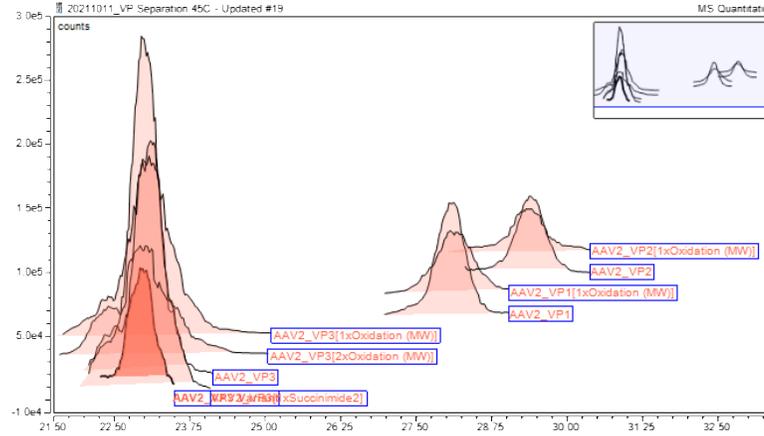
Data Processing Parameter Optimization (Sf9 Derived AAVs)



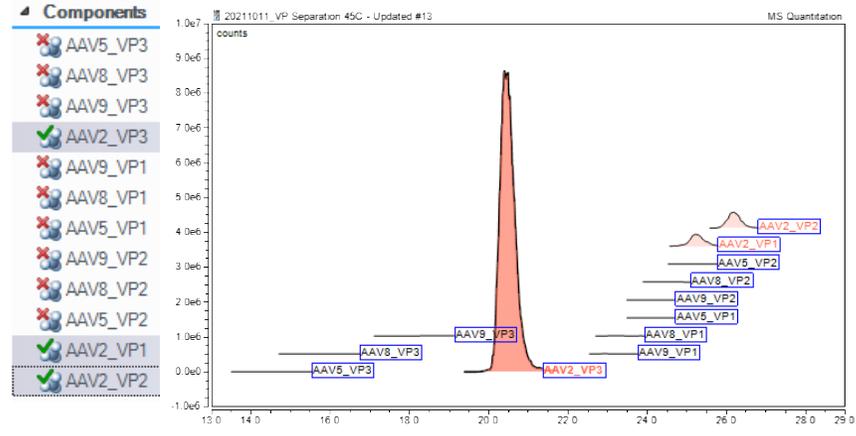
Report Generation



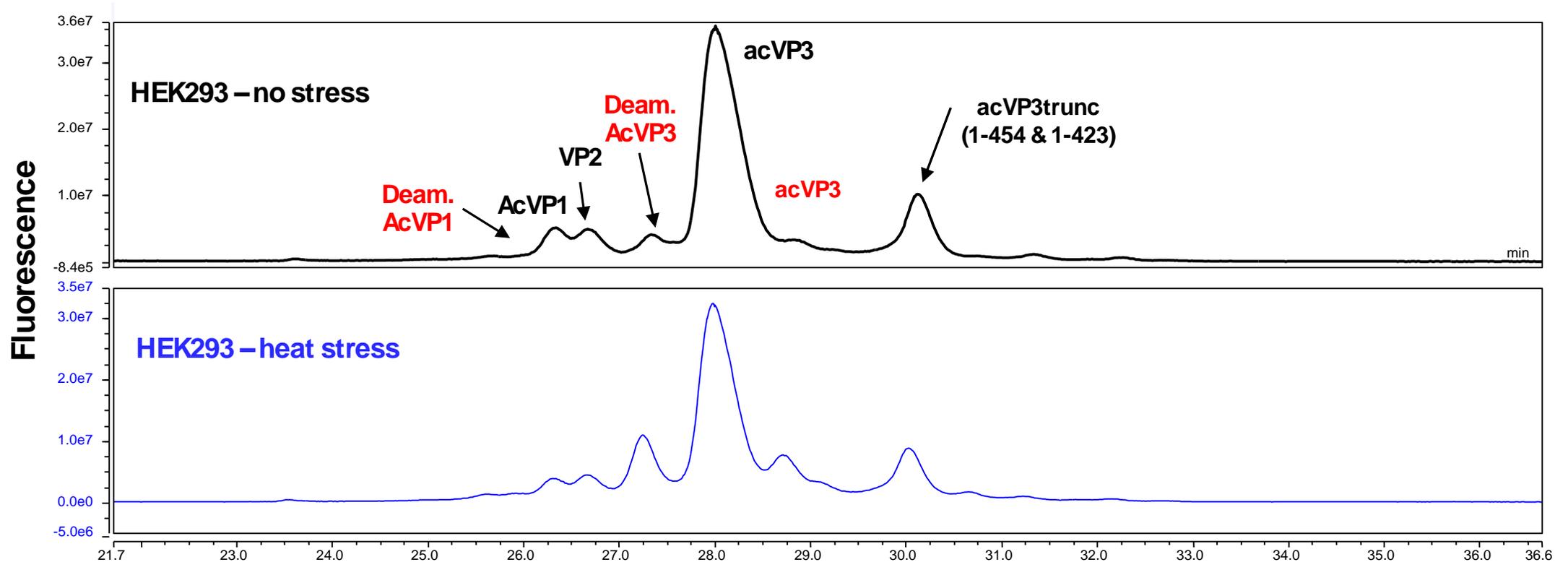
Specific Serotype PTM Monitoring



Method Validation (HEK Derived AAV)



RP Separation for Detection of Deamidation Events

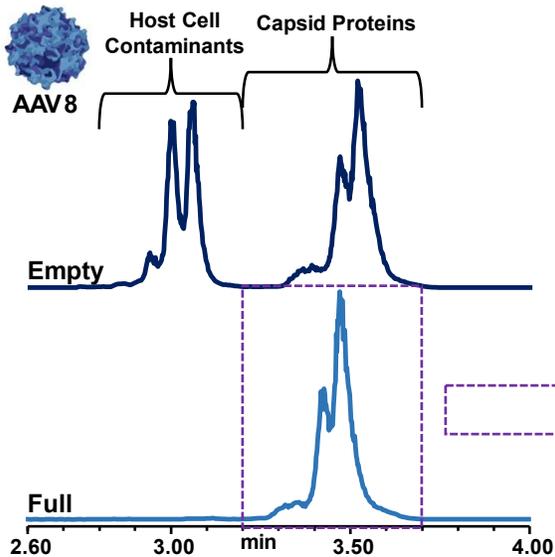


While the HILIC separation of VP's works well, for modifications such as deamidation events, HILIC does not have the necessary selectivity. Reversed-phase separation on C4 enables efficient separation of deamidated forms of the viral proteins.

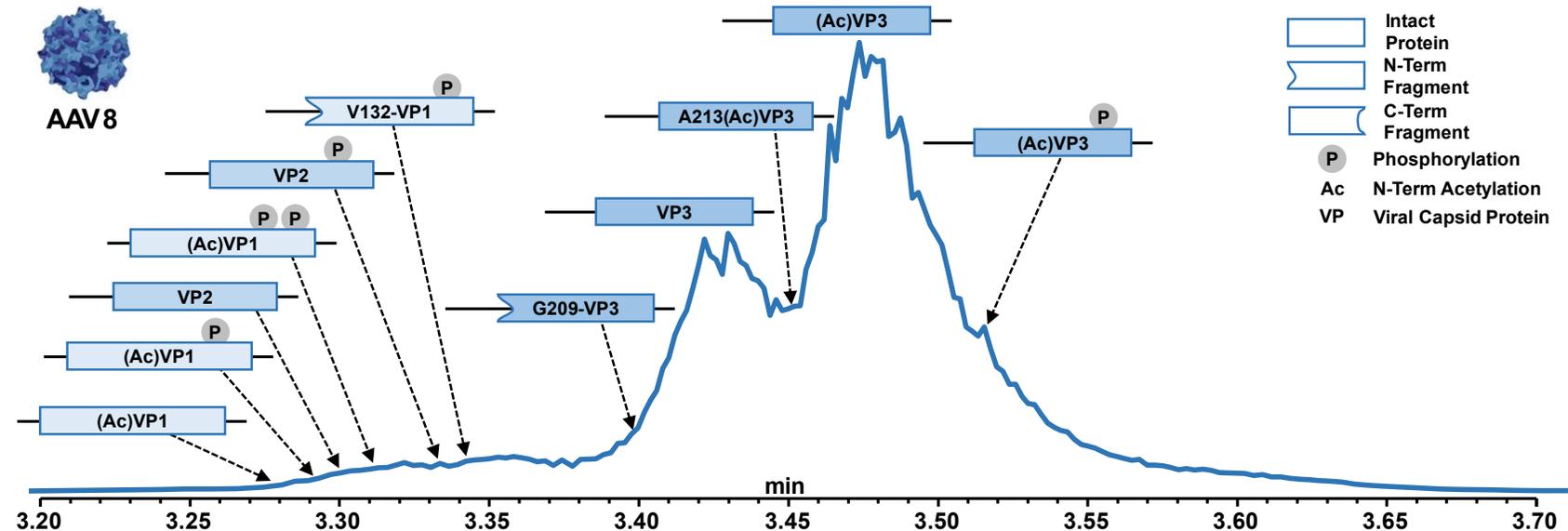
Viral Protein Separation using MCE-MS



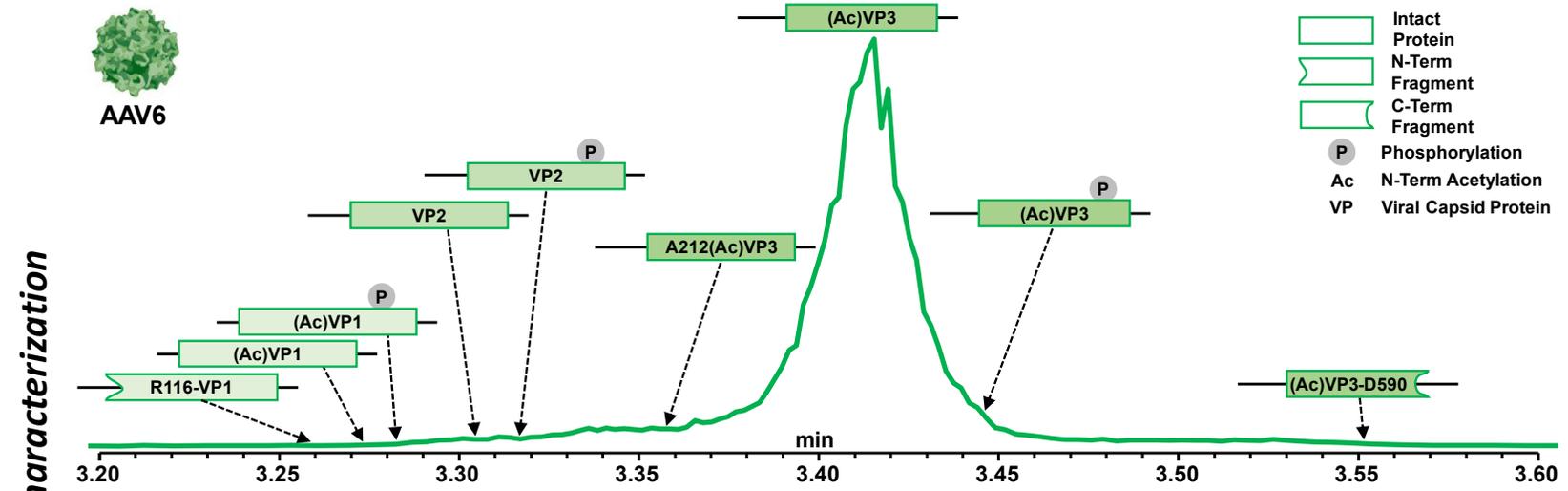
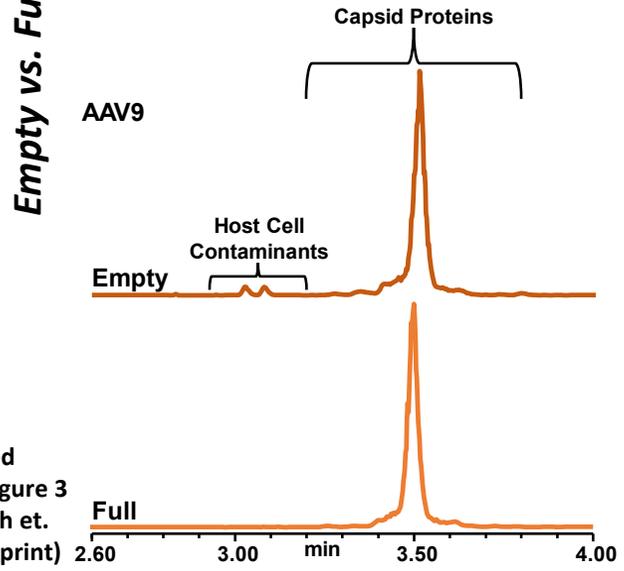
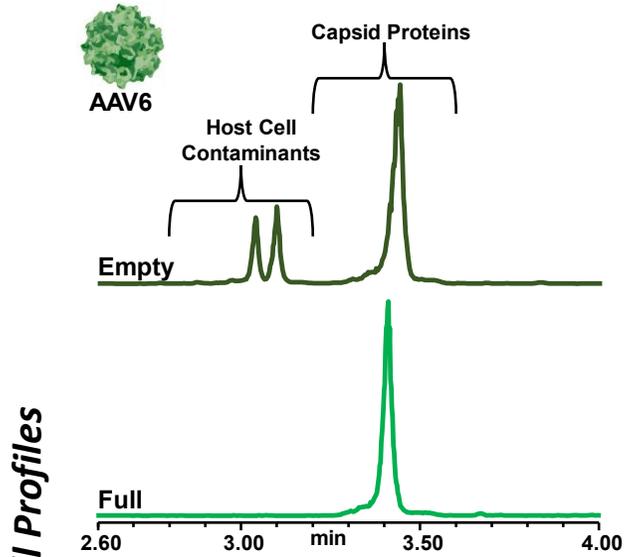
Empty vs. Full Profiles



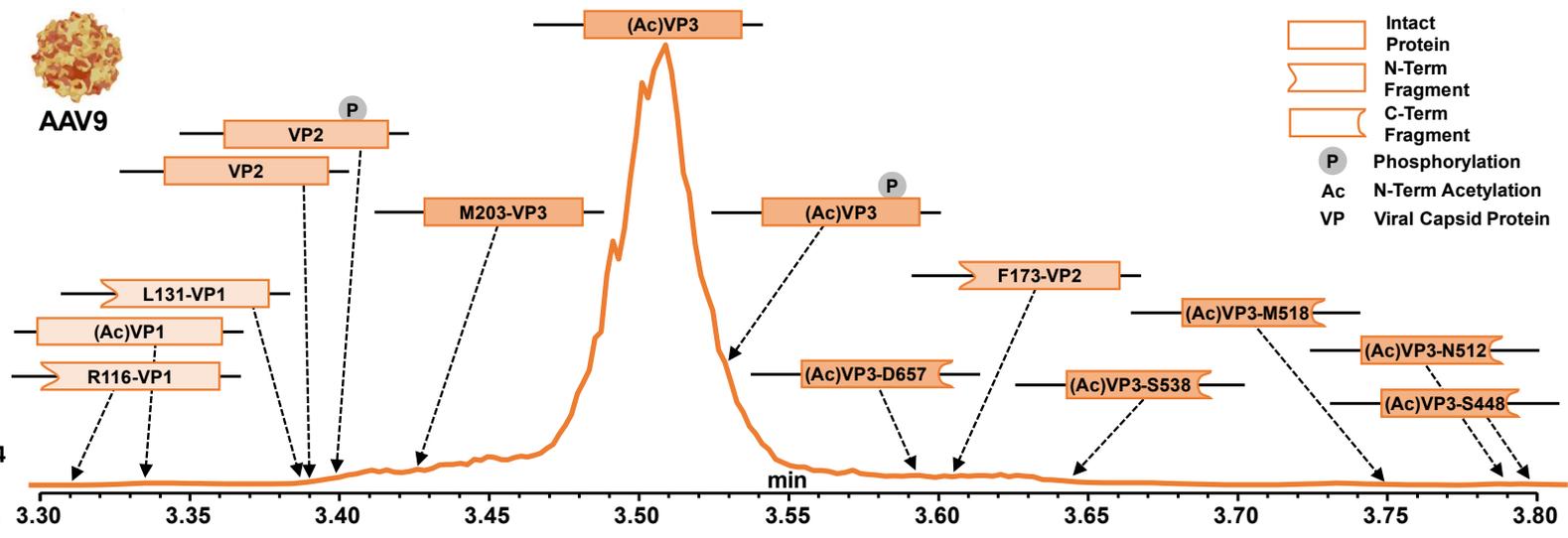
VP Proteoform Characterization



Broad Applicability of ZipChip Platform



- Intact Protein
- N-Term Fragment
- C-Term Fragment
- Phosphorylation
- N-Term Acetylation
- Viral Capsid Protein



- Intact Protein
- N-Term Fragment
- C-Term Fragment
- Phosphorylation
- N-Term Acetylation
- Viral Capsid Protein

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

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

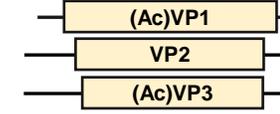


Detected VP Proteoforms and Fragments

Start of AAV Sequence



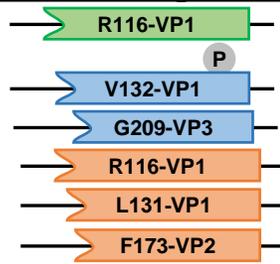
Expected VPs



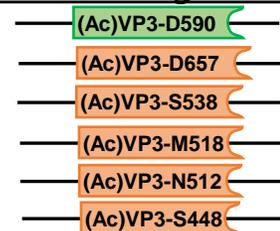
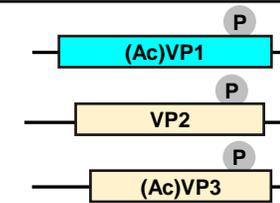
VP3 Variant



N-Term Fragments



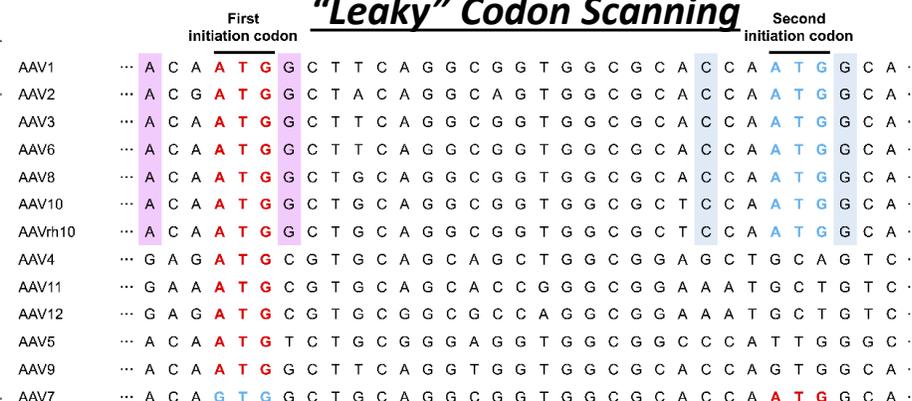
VPs with additional PTMs C-Term Fragments



VP3 Variant Generation

Serotypes	N-terminal region		DP sequence	DG sequence	DP sequence	AAV1
	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	... A C A A T G G C T T C A G G C G G T G G C G C A C C A A T G G C A ...
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	... A C G A T G G C T A C A G G C A G T G G C G C A C C A A T G G C A ...
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	... A C A A T G G C T T C A G G C G G T G G C G C A C C A A T G G C A ...
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	... A C A A T G G C T T C A G G C G G T G G C G C A C C A A T G G C A ...
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	... A C A A T G G C T G C A G G C G G T G G C G C A C C A A T G G C A ...
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	... A C A A T G G C T G C A G G C G G T G G C G C T C C A A T G G C A ...
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	... A C A A T G G C T G C A G G C G G T G G C G C T C C A A T G G C A ...
AAV4	M R A A A G G A A V	M A	N L P T	T D G H	A N P A	... G A G A T G C G T G C A G C A G C T G G C G G A G C T G C A G T C ...
AAV11	M R A A P G G N A V	M A	T A P I	A D G H	A N P A	... G A A A T G C G T G C A G C A C C G G G C G G A A A T G C T G T C ...
AAV12	M R A A P G G N A V	M A	T A P H	T D G H	A N P N	... G A G A T G C G T G C G G C G C C A G G C G G A A A T G C T G T C ...
AAV5	M S A G G G P L G	M A	T A P A	T G A H	G N I -	... A C A A T G T C T C G G G A G G T G G C G G C C A T T G G G C ...
AAV9	M A S G G G A P V A	M A	A Q A Q	T D G N	A D P P	... A C A A T G G C T T C A G G T G G T G G C G C A C C A G T G G C A ...
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	... A C A G T G C T G C A G G C G G T G G C G C A C C A A T G G C A ...

"Leaky" Codon Scanning

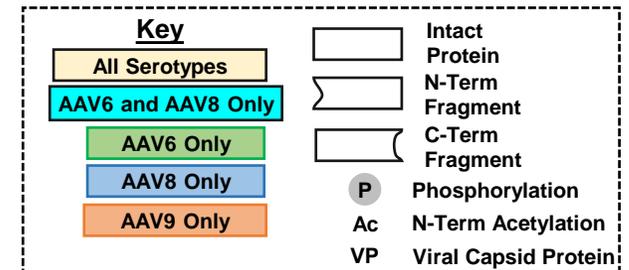


Unexpected VPs



Potential Causes of Fragments

- Baculoviral cathepsin
- Immune response
- Acidic conditions



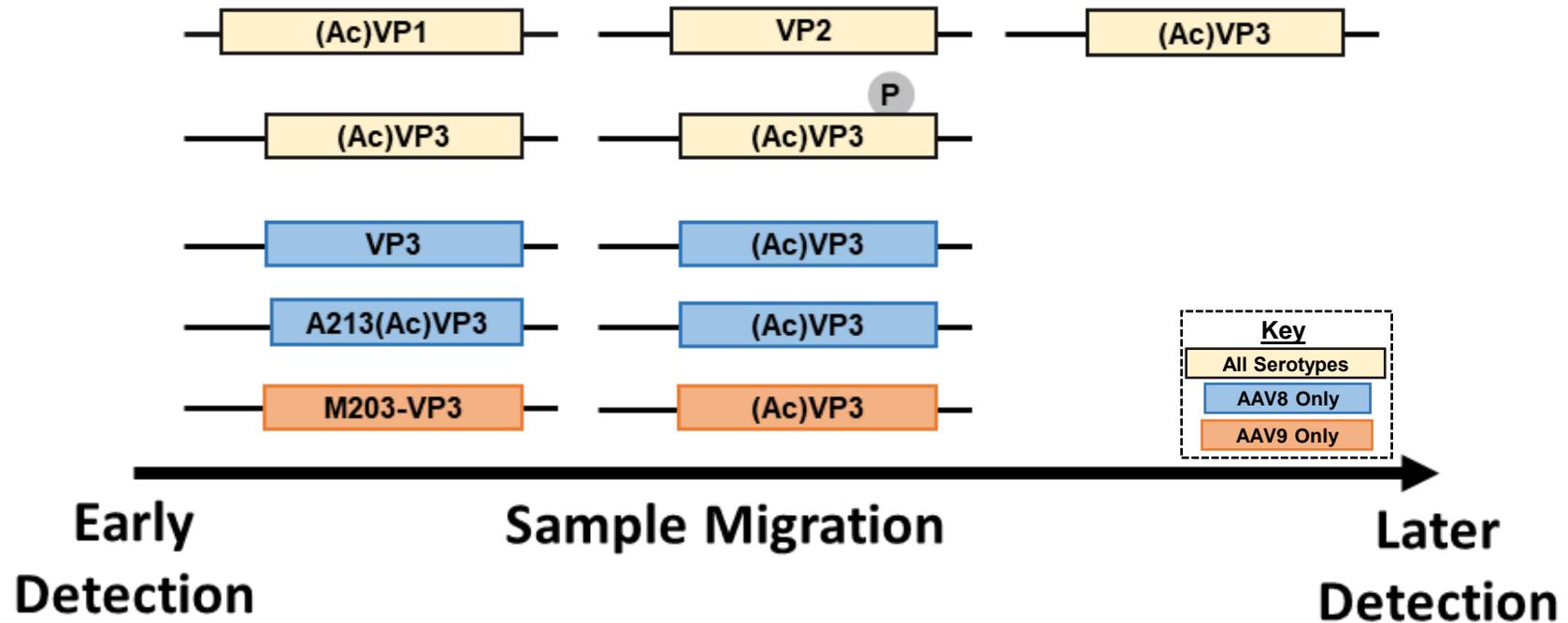
Adapted from Figure 5a of Oyama et al. (2021)
<https://www.liebertpub.com/doi/10.1089/hum.2021.009>

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



Influence of Modifications on VP Migration

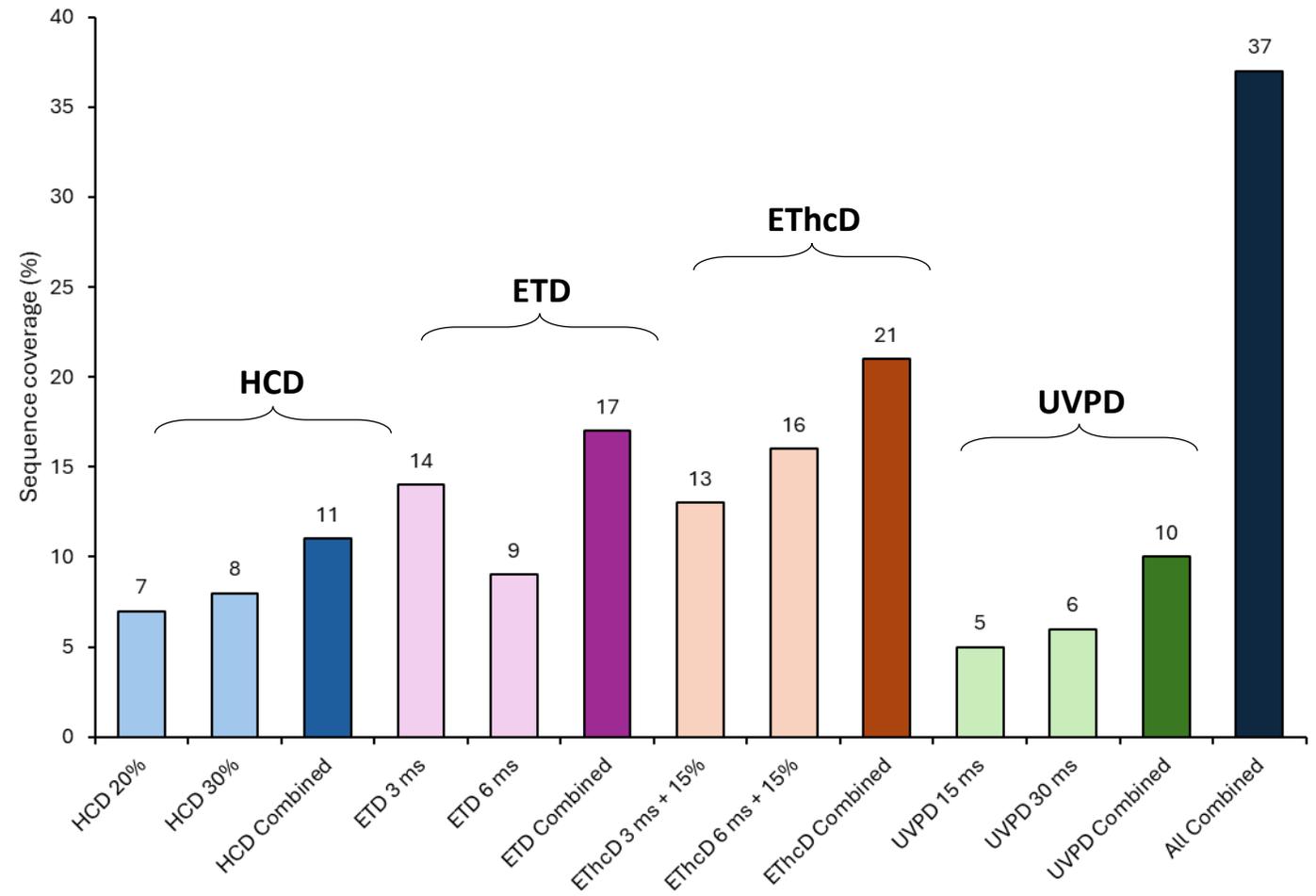
Serotype	Amino Acid Sequence	Viral Protein	Capsids (Empty/Full)	Relative Abundance (%)	Migration Time (min)	Theoretical Net Charge at pH 2.4	Theoretical Charge to Mass ratio
AAV8	A2(Ac)-L738	(Ac)VP1 + 2xP	Empty	0.13	3.336	+73.996	9.04×10^{-4}
			Full	0.08	3.311		
		(Ac)VP1 + 1xP	Empty	0.62	3.322	+74.937	9.17×10^{-4}
	Full	0.41	3.291				
	(Ac)VP1	Empty	0.42	3.310	+75.878	9.29×10^{-4}	
		Full	0.21	3.278			
	V132-L738	V132-VP1 + 1xP Fragment	Empty	0.11	3.370	+59.268	8.80×10^{-4}
	Full	0.07	3.339				
	A139-L738	VP2 + 1xP	Empty	1.27	3.374	+58.286	8.75×10^{-4}
			Full	0.89	3.332		
VP2	Empty	0.57	3.338	+59.226	8.90×10^{-4}		
	Full	0.38	3.299				
A205(Ac)-L738	(Ac)VP3 + 1xP	Empty	6.64	3.546	+48.375	8.08×10^{-4}	
		Full	6.85	3.511			
	(Ac)VP3	Empty	100.00	3.521	+49.316	8.25×10^{-4}	
Full	100.00	3.485					
VP3	Empty	49.75	3.468	+50.316	8.42×10^{-4}		
	Full	45.29	3.431				
G209-L738	G208-VP3 Fragment	Empty	3.40	3.441	+50.316	8.46×10^{-4}	
Full	2.61	3.402					
A213(Ac)-L738	(Ac)VP3 Variant	Empty	4.46	3.484	+49.316	8.33×10^{-4}	
		Full	4.94	3.450			
Serotype	Amino Acid Sequence	Viral Protein	Capsids (Empty/Full)	Relative Abundance (%)	Migration Time (min)	Theoretical Net Charge at pH 2.4	Theoretical Charge to Mass ratio
AAV9	A2(Ac)-L736	(Ac)VP1	Empty	0.11	3.328	+76.860	9.45×10^{-4}
	Full	0.02	3.335				
	R116-L736	R116-VP1 Fragment 1	Empty	0.22	3.319	+64.161	9.33×10^{-4}
			Full	-	-		
	L131-L736	L131-VP1 Fragment 2	Empty	0.20	3.383	+60.170	8.97×10^{-4}
			Full	0.04	3.387		
	A139-L736	VP2 + 1xP	Empty	-	3.398	+58.247	8.79×10^{-4}
			Full	0.03	-		
	VP2	Empty	0.67	3.389	+59.188	8.94×10^{-4}	
		Full	0.33	3.390			
	F173-L736	F173-VP2 Fragment	Empty	0.68	3.597	+52.226	8.33×10^{-4}
			Full	0.23	3.605		
	M203-L736	M203-VP3	Empty	0.43	3.420	+52.286	8.74×10^{-4}
			Full	0.41	3.418		
	A204(Ac)-L736	(Ac)VP3 + 1xP	Empty	7.68	3.533	+50.345	8.42×10^{-4}
			Full	9.79	3.531		
(Ac)VP3	Empty	100.00	3.498	+51.286	8.59×10^{-4}		
	Full	100.00	3.492				
A204(Ac)-D657	(Ac)VP3-D657 Fragment	Empty	0.09	3.588	+42.363	8.39×10^{-4}	
		Full	0.11	3.593			
A204(Ac)-S538	(Ac)VP3-S538 Fragment	Empty	0.37	3.645	+30.547	8.13×10^{-4}	
		Full	0.42	3.647			
A204(Ac)-M518	(Ac)VP3-M518 Fragment	Empty	0.13	3.745	+27.594	7.80×10^{-4}	
		Full	0.15	3.750			
A204(Ac)-N512	(Ac)VP3-N512 Fragment	Empty	0.10	3.789	+26.589	7.66×10^{-4}	
		Full	0.09	3.793			
A204(Ac)-S448	(Ac)VP3-S448 Fragment	Empty	0.58	3.800	+21.594	7.77×10^{-4}	
		Full	0.20	3.804			



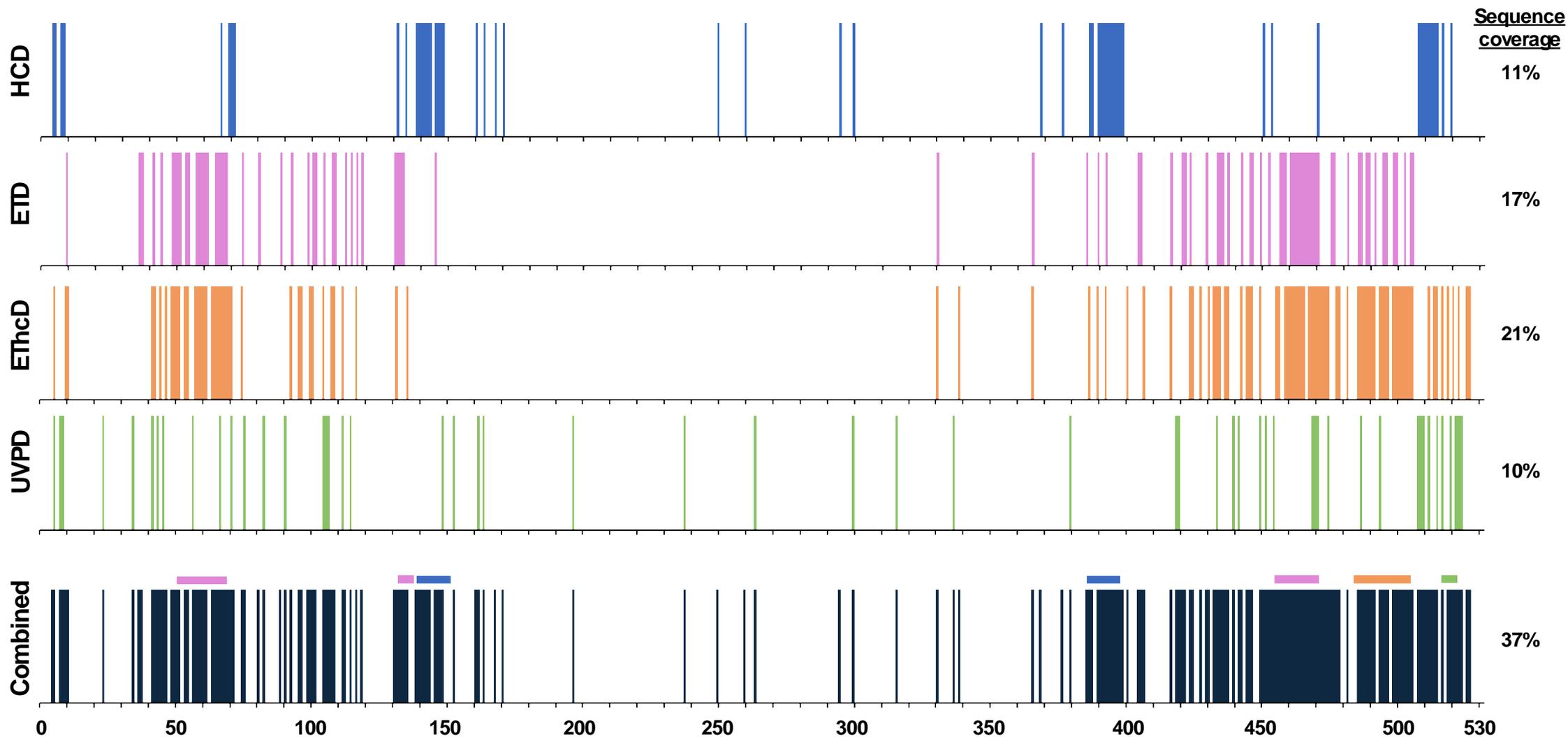
ProtPi (<https://www.protpi.ch/Calculator/ProteinTool>) used to calculate Theoretical Net Charge of VPs at pH 2.4 (pH of BGE)

Top-Down MS/MS Sequencing of Intact VP's – Focus on VP3

- ❑ Sequence degeneracy of VP's complicates annotation of PTMs.
- ❑ Rather than use peptide mapping, top-down MS/MS sequencing on Orbitrap Eclipse was investigated using multiple ion activation strategies.
- ❑ EThcD fragmentation resulted in highest individual coverage.
- ❑ Combining all ion activation strategies, sequence coverage resulted in nice N- and C-terminal fragmentation.



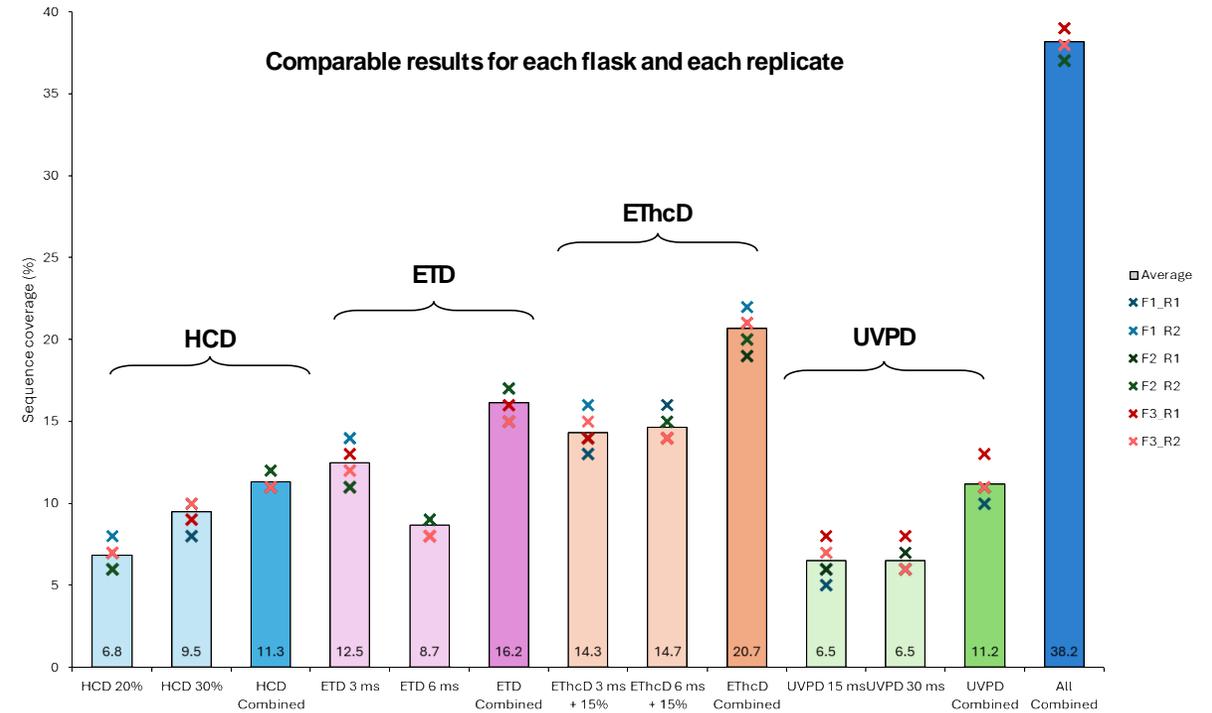
Combined Top-Down MS/MS of VP3 on Orbitrap Eclipse



Top-down MS/MS Data Sequence Map

Data from barcode plot mapped on to the sequence of VP3 demonstrating nice N- and C-terminal fragmentation.

N **A** S G G|G|A|P V|A|D|N|N E G A D G|V G S S S G N|W| 25
 26 H C D S Q W L G D R V I T T S T R|T|W|A|L|P|T|Y|N| 50
 51 N|H|L|Y|K|Q|I|S|N|S|T|S|G|G|S|S|N|D|N|A|Y|F|G|Y|S| 75
 76 T|P|W|G|Y|F|D|F|N|R|F|H|C|H|F|S|P|R|D|W|Q|R|L|I|N| 100
 101 N|N|W|G|F|R|P|K|R|L|N|F|K|L|F|N|I|Q|V|K|E|V|T|D|N| 125
 126 N G V K T I|A|N|N|L|T|S T|V|Q|V|F|T|D|S|D|Y|Q|L|P| 150
 151 Y V L G S A H E G C L|P|P F|P A D V|F M|I|P Q Y G| 175
 176 Y L T L N D G S Q A V G R S S F Y C L E Y F P S Q| 200
 201 M L R T G N N F Q F S Y E F E N V P F H S S Y A H| 225
 226 S Q S L D R|L M N P L I D Q Y L Y Y L S K T I N G| 250
 251|S G Q|N Q Q T L K F S V A G P S N M A V Q G R N Y| 275
 276 I P G P S Y R Q Q R V S T T V T Q N N N S E|F A|W| 300
 301|P G A S S W A L N G R N S L M|N P G|P A M A S H|K| 325
 326 E G E D R F|F P L S G S|L I F G K Q G T G R D N V| 350
 351|D A D K V M I T N E E E I K T T|N P V|A T E S Y G| 375
 376 Q V A T N H Q S A Q|A|Q|A|Q|T|G|W|V|Q|N|Q|G|I|L|P| 400
 401 G|M V W|Q|D|R D V Y L Q|G P I W A|K|I P|H T|D G|N| 425
 426|F H P|S|P|L|M G|G|F|G|M|K|H P P|P Q|I L I|K|N|T P| 450
 451|V P|A|D|P|P|T|A|F|N|K|D|K|L|N|S|F|I|T|Q|Y|S|T|G|Q| 475
 476|V S V|E I|E W|E L|Q K|E|N|S|K|R|W|N P|E|I|Q|Y|T|S| 500
 501|N|Y|Y|K|S|N|N V|E|F|A|V|N|T|E|G V|Y S|E|P|R|P|I|G| 525
 526|T|R|Y L T R N L C



Reproducibility of fragmentation behaviour investigated using three different preparations of HEK293 derived AAV9 and replicate instrumental analysis.

Reproducibility of fragmentation was found to be high resulting in the same pattern each time for biological and technical replicate measurements

Top-Down MS/MS Facilitated Site Specific PTM Annotation

N-term Ac – 40%

```
N S G G G A P V A D N N E G A D G V G S S S G N W 25
26 H C D S Q W L G D R V I T T S T R T W A L P T Y N 50
51 N H L Y K Q I S N S T S G G S S N D N A Y F G Y S 75
76 T P W G Y F D F N R F H C H F S P R D W Q R L I N 100
101 N N W G F R P K R L N F K L F N I Q V K E V T D N 125
126 N G V K T I A N N L T S T V Q V F T D S D Y Q L P 150
151 Y V L G S A H E G C L P P F P A D V F M I P Q Y G 175
176 Y L T L N D G S Q A V G R S S F Y C L E Y F P S Q 200
201 M L R T G N N F Q F S Y E F E N V P F H S S Y A H 225
226 S Q S L D R L M N P L I D Q Y L Y Y L S K T I N G 250
251 S G Q N Q Q T L K F S V A G P S N M A V Q G R N Y 275
276 I P G P S Y R Q Q R V S T T V T Q N N N S E F A W 300
301 P G A S S W A L N G R N S L M N P G P A M A S H K 325
326 E G E D R F F P L S G S L I F G K Q G T G R D N V 350
351 D A D K V M I T N E E E I K T T N P V A T E S Y G 375
376 Q V A T N H Q S A Q A Q A Q A Q T G W V Q N Q G I L P 400
401 G M V W Q D R D V Y L Q G P I W A K I P H T D G N 425
426 F H P S P L M G G F G M K H P P P Q I L I K N T P 450
451 V P A D P P T A F N K D K L N S F I I T Q Y L S T G Q 475
476 V S V E I E W E L Q K E N S K R I W N P E I I Q Y T S 500
501 N Y Y K S N N V E F A V N I T E G V Y S E P R P I G 525
526 T R I Y L T R N L C
```

K108 Ac – 31%

```
N A S G G G A P V A D N N E G A D G V G S S S G N W 25
26 H C D S Q W L G D R V I T T S T R T W A L P T Y N 50
51 N H L Y K Q I S N S T S G G S S N D N A Y F G Y S 75
76 T P W G Y F D F N R F H C H F S P R D W Q R L I N 100
101 N N W G F R P K R L N F K L F N I Q V K E V T D N 125
126 N G V K T I A N N L T S T V Q V F T D S D Y Q L P 150
151 Y V L G S A H E G C L P P F P A D V F M I P Q Y G 175
176 Y L T L N D G S Q A V G R S S F Y C L E Y F P S Q 200
201 M L R T G N N F Q F S Y E F E N V P F H S S Y A H 225
226 S Q S L D R L M N P L I D Q Y L Y Y L S K T I N G 250
251 S G Q N Q Q T L K F S V A G P S N M A V Q G R N Y 275
276 I P G P S Y R Q Q R V S T T V T Q N N N S E F A W 300
301 P G A S S W A L N G R N S L M N P G P A M A S H K 325
326 E G E D R F F P L S G S L I F G K Q G T G R D N V 350
351 D A D K V M I T N E E E I K T T N P V A T E S Y G 375
376 Q V A T N H Q S A Q A Q A Q A Q T G W V Q N Q G I L P 400
401 G M V W Q D R D V Y L Q G P I W A K I P H T D G N 425
426 F H P S P L M G G F G M K H P P P Q I L I K N T P 450
451 V P A D P P T A F N K D K L N S F I I T Q Y L S T G Q 475
476 V S V E I E W E L Q K E N S K R I W N P E I I Q Y T S 500
501 N Y Y K S N N V E F A V N I T E G V Y S E P R P I G 525
526 T R I Y L T R N L C
```

K504 Ac – 11%

```
N A S G G G A P V A D N N E G A D G V G S S S G N W 25
26 H C D S Q W L G D R V I T T S T R T W A L P T Y N 50
51 N H L Y K Q I S N S T S G G S S N D N A Y F G Y S 75
76 T P W G Y F D F N R F H C H F S P R D W Q R L I N 100
101 N N W G F R P K R L N F K L F N I Q V K E V T D N 125
126 N G V K T I A N N L T S T V Q V F T D S D Y Q L P 150
151 Y V L G S A H E G C L P P F P A D V F M I P Q Y G 175
176 Y L T L N D G S Q A V G R S S F Y C L E Y F P S Q 200
201 M L R T G N N F Q F S Y E F E N V P F H S S Y A H 225
226 S Q S L D R L M N P L I D Q Y L Y Y L S K T I N G 250
251 S G Q N Q Q T L K F S V A G P S N M A V Q G R N Y 275
276 I P G P S Y R Q Q R V S T T V T Q N N N S E F A W 300
301 P G A S S W A L N G R N S L M N P G P A M A S H K 325
326 E G E D R F F P L S G S L I F G K Q G T G R D N V 350
351 D A D K V M I T N E E E I K T T N P V A T E S Y G 375
376 Q V A T N H Q S A Q A Q A Q A Q T G W V Q N Q G I L P 400
401 G M V W Q D R D V Y L Q G P I W A K I P H T D G N 425
426 F H P S P L M G G F G M K H P P P Q I L I K N T P 450
451 V P A D P P T A F N K D K L N S F I I T Q Y L S T G Q 475
476 V S V E I E W E L Q K E N S K R I W N P E I I Q Y T S 500
501 N Y Y K S N N V E F A V N I T E G V Y S E P R P I G 525
526 T R I Y L T R N L C
```

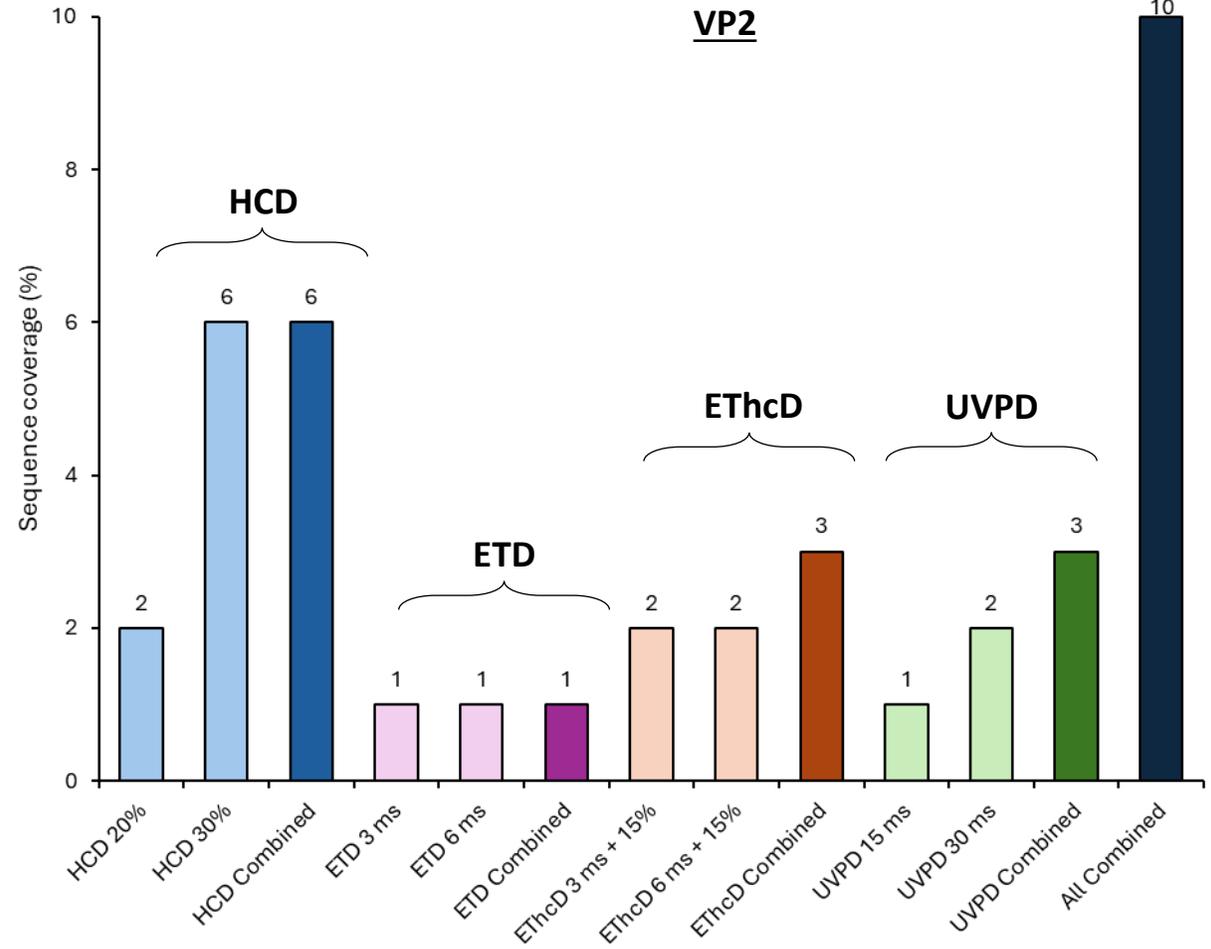
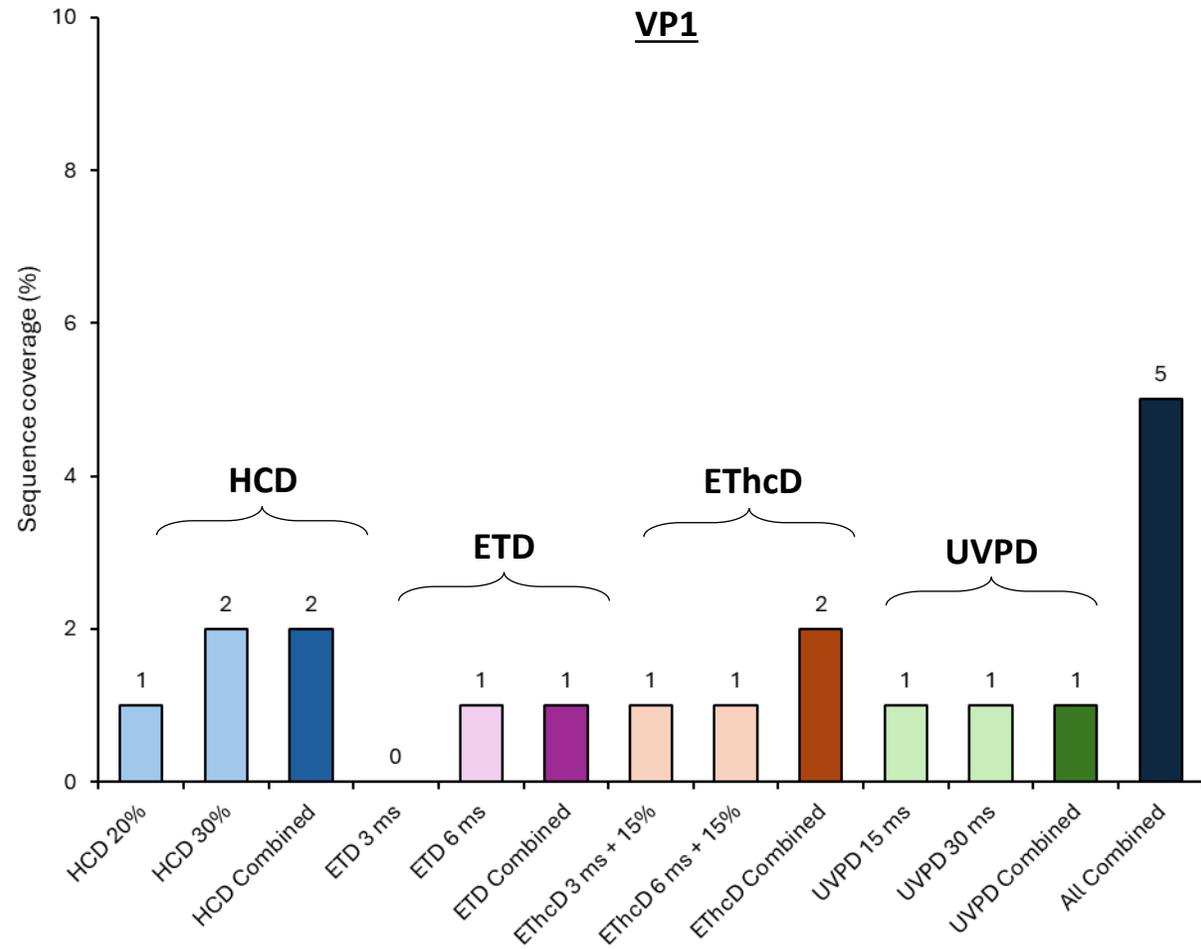
K55 Ac – 37%

```
N A S G G G A P V A D N N E G A D G V G S S S G N W 25
26 H C D S Q W L G D R V I T T S T R T W A L P T Y N 50
51 N H L Y K Q I S N S T S G G S S N D N A Y F G Y S 75
76 T P W G Y F D F N R F H C H F S P R D W Q R L I N 100
101 N N W G F R P K R L N F K L F N I Q V K E V T D N 125
126 N G V K T I A N N L T S T V Q V F T D S D Y Q L P 150
151 Y V L G S A H E G C L P P F P A D V F M I P Q Y G 175
176 Y L T L N D G S Q A V G R S S F Y C L E Y F P S Q 200
201 M L R T G N N F Q F S Y E F E N V P F H S S Y A H 225
226 S Q S L D R L M N P L I D Q Y L Y Y L S K T I N G 250
251 S G Q N Q Q T L K F S V A G P S N M A V Q G R N Y 275
276 I P G P S Y R Q Q R V S T T V T Q N N N S E F A W 300
301 P G A S S W A L N G R N S L M N P G P A M A S H K 325
326 E G E D R F F P L S G S L I F G K Q G T G R D N V 350
351 D A D K V M I T N E E E I K T T N P V A T E S Y G 375
376 Q V A T N H Q S A Q A Q A Q A Q T G W V Q N Q G I L P 400
401 G M V W Q D R D V Y L Q G P I W A K I P H T D G N 425
426 F H P S P L M G G F G M K H P P P Q I L I K N T P 450
451 V P A D P P T A F N K D K L N S F I I T Q Y L S T G Q 475
476 V S V E I E W E L Q K E N S K R I W N P E I I Q Y T S 500
501 N Y Y K S N N V E F A V N I T E G V Y S E P R P I G 525
526 T R I Y L T R N L C
```

K461 Ac – 15%

```
N A S G G G A P V A D N N E G A D G V G S S S G N W 25
26 H C D S Q W L G D R V I T T S T R T W A L P T Y N 50
51 N H L Y K Q I S N S T S G G S S N D N A Y F G Y S 75
76 T P W G Y F D F N R F H C H F S P R D W Q R L I N 100
101 N N W G F R P K R L N F K L F N I Q V K E V T D N 125
126 N G V K T I A N N L T S T V Q V F T D S D Y Q L P 150
151 Y V L G S A H E G C L P P F P A D V F M I P Q Y G 175
176 Y L T L N D G S Q A V G R S S F Y C L E Y F P S Q 200
201 M L R T G N N F Q F S Y E F E N V P F H S S Y A H 225
226 S Q S L D R L M N P L I D Q Y L Y Y L S K T I N G 250
251 S G Q N Q Q T L K F S V A G P S N M A V Q G R N Y 275
276 I P G P S Y R Q Q R V S T T V T Q N N N S E F A W 300
301 P G A S S W A L N G R N S L M N P G P A M A S H K 325
326 E G E D R F F P L S G S L I F G K Q G T G R D N V 350
351 D A D K V M I T N E E E I K T T N P V A T E S Y G 375
376 Q V A T N H Q S A Q A Q A Q A Q T G W V Q N Q G I L P 400
401 G M V W Q D R D V Y L Q G P I W A K I P H T D G N 425
426 F H P S P L M G G F G M K H P P P Q I L I K N T P 450
451 V P A D P P T A F N K D K L N S F I I T Q Y L S T G Q 475
476 V S V E I E W E L Q K E N S K R I W N P E I I Q Y T S 500
501 N Y Y K S N N V E F A V N I T E G V Y S E P R P I G 525
526 T R I Y L T R N L C
```

What about VP1 and VP2?



Sequence Coverage Maps

VP2 (10% sequence coverage)

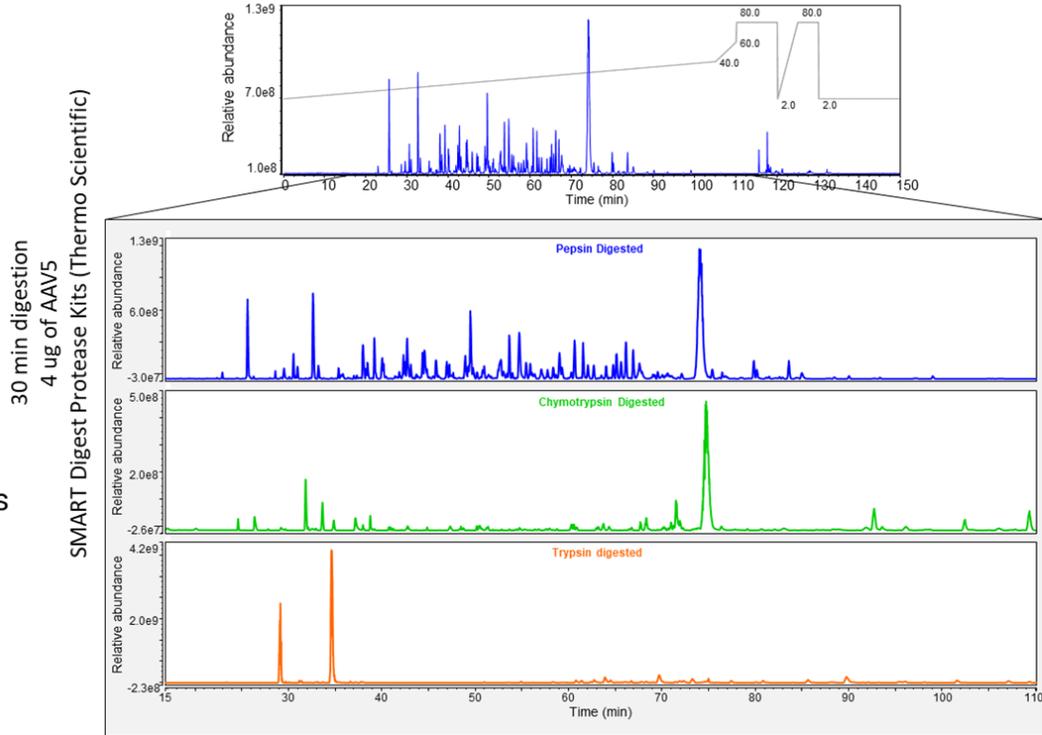
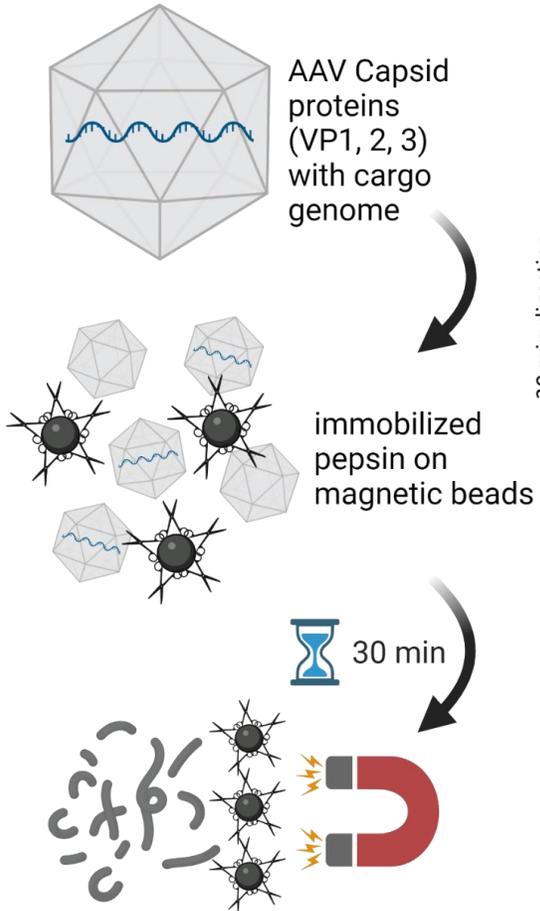
```
N  A P G K K R P V E Q S P Q E P D S S A G I G K S G 25
26 A Q P A K R L N F G Q T G D T E S V P D P Q P I 50
51 G E P P A A P S G V G S L T M A S G G G A P V A D 75
76 N N E G A D G V G S S S G N W H C D S Q W L G D R 100
101 V I T T S T R T W A L P T Y N N H L Y K Q I S N S 125
126 T S G G S S N D N A Y F G Y S T P W G Y F D F N R 150
151 F H C H F S P R D W Q R L I N N N W G F R P K R L 175
176 N F K L F N I Q V K E V T D N N G V K T I A N N L 200
201 T S T V Q V F T D S D Y Q L P Y V L G S A H E G C 225
226 L P P F P A D V F M I P Q Y G Y L T L N D G S Q A 250
251 V G R S S F Y C L E Y F P S Q M L R T G N N F Q F 275
276 S Y E F E N V P F H S S Y A H S Q S L D R L M N P 300
301 L I D Q Y L Y Y L S K T I N G S G Q N Q Q T L K F 325
326 S V A G P S N M A V Q G R N Y I P G P S Y R Q Q R 350
351 V S T T V T Q N N N S E F A W P G A S S W A L N G 375
376 R N S L M N P G P A M A S H K E G E D R F F P L S 400
401 G S L I F G K Q G T G R D N V D A D K V M I T N E 425
426 E E I K T T N P V A T E S Y G Q V A T N H Q S A Q 450
451 A Q A Q T G W V Q N Q G I L L P G M V W Q D R D V Y 475
476 L Q G P I W A K I P H T D G N F H P S P L M G G F 500
501 G M K H P P P Q I L I K N T P V P A D P P T A F N 525
526 K D K L N S F I T Q Y S T G Q V S V E I E W E L Q 550
551 K E N S K R W N P E I Q Y T S N Y Y K S N N V E F 575
576 A V N T E G V Y S E P R P I G T R Y L T R N L C
```

VP1 (5% sequence coverage)

```
N  A A D G Y L P D W L E D N L S E G I R E W W A L K 25
26 P G A P Q P K A N Q Q H Q D N A R G L V L P G Y K 50
51 Y L G P G N G L D K G E P V N A A D A A A L E H D 75
76 K A Y D Q Q L K A G D N P Y L K Y N H A D A E F Q 100
101 E R L K E D T S F G G N L G R A V F Q A K K R L L 125
126 E P L G L V E E A A K T A P G K K R P V E Q S P Q 150
151 E P D S S A G I G K S G A Q P A K K R L N F G Q T 175
176 G D T E S V P D P Q P I G E P P A A P S G V G S L 200
201 T M A S G G G A P V A D N N E G A D G V G S S S G 225
226 N W H C D S Q W L G D R V I T T S T R T W A L P T 250
251 Y N N H L Y K Q I S N S T S G G S S N D N A Y F G 275
276 Y S T P W G Y F D F N R F H C H F S P R D W Q R L 300
301 I N N N W G F R P K R L N F K L F N I Q V K E V T 325
326 D N N G V K T I A N N L T S T V Q V F T D S D Y Q 350
351 L P Y V L G S A H E G C L P P F P A D V F M I P Q 375
376 Y G Y L T L N D G S Q A V G R S S F Y C L E Y F P 400
401 S Q M L R T G N N F Q F S Y E F E N V P F H S S Y 425
426 A H S Q S L D R L M N P L I D Q Y L Y Y L S K T I 450
451 N G S G Q N Q Q T L K F S V A G P S N M A V Q G R 475
476 N Y I P G P S Y R Q Q R V S T T V T Q N N N S E F 500
501 A W P G A S S W A L N G R N S L M N P G P A M A S 525
526 H K E G E D R F F P L S G S L I F G K Q G T G R D 550
551 N V D A D K V M I T N E E E I K T T N P V A T E S 575
576 Y G Q V A T N H Q S A Q A Q A Q T G W V Q N Q G I 600
601 L P G M V W Q D R D V Y L Q G P I W A K I P H T D 625
626 G N F H P S P L M G G F G M K H P P P Q I L I K N 650
651 T P V P A D P P T A F N K D K L N S F I T Q Y S T 675
676 G Q V S V E I E W E L Q K E N S K R W N P E I Q Y 700
701 T S N Y Y K S N N V E F A V N T E G V Y S E P R P 725
726 I G T R Y L T R N L C
```

Lower abundance of VP1 and 2 resulted in lower sequence coverage. Interestingly, most fragmentation observed in the N-terminal region. Further investigation on-going.

AAV Peptide Mapping Workflow



VP1
MSFVDHPPDWLEEVGEGLREFLGLEAGPPKPKPNQQHQDQARGLVLPGYNYLGPNGNGLDRG
 EPVNRADAVAREHDISYNEQLEAGDNPYLKYNHADAEFQEKLADDTSTFGGNLKGAVFQAKKR
 VP2
 VLEPFGLVEEGAKTAPTGKRIDDHFPKRKKARTEEDSKPSTSSDAEAGPSGSQQLQIPAPASS
 VP3
 LGADTMSAGGGGGLGDNNQGADGVGNASGDWHCDSTWMDRVRTKSTRTWLPSYNNHQ
 YREIKSGSVDGSNANAYFGYSTPWGYFDFNRFHSHWSPRDWQRLINNYWGFRRSLRVKIFN
 IQVKEVTVDQSTTTIANLLTSTVQVFTDDDYQLPYVVGNTEGCLPAFPQVFTLPQYGYATLN
 RDNTENPTERSSSFCLEYFPSKMLRTGNNFEFTYNFEEVPFHSSFAPSQNLFKLANPLVDQYLY
 RFVSTNNTGGVQFNKNLAGRYANTYKNWFPGPMGRTQGWNLGSQVNRASVSFAFATTNRMEL
 EGASYQVPPQPNGMTNNLQGSNTYALENTMIFNSQPANPGTTATYLEGNMLITSESETQPVNR
 VAYNVGGQMATNNQSSTTAPATGTYNLQEIVPGSVWMERDVYLQGPWAKIPETGAHFHPSPA
 MGGFGLKHPPMMLIKNTPVPGNITSFSDVPVSSFITQYSTGQVTVEMEWELKKENSKRWNP
 EIQTNNYNDPQFVDFAPDSTGEYRTRPIGTRYLTRPL

Efficient digestion of AAV using pepsin. Immobilised protease on magnetic beads enabled tight time control when combined with KingFisher Duo Prime Automation Station.



HCP Analysis Using Orbitrap Astral LC-MS

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

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

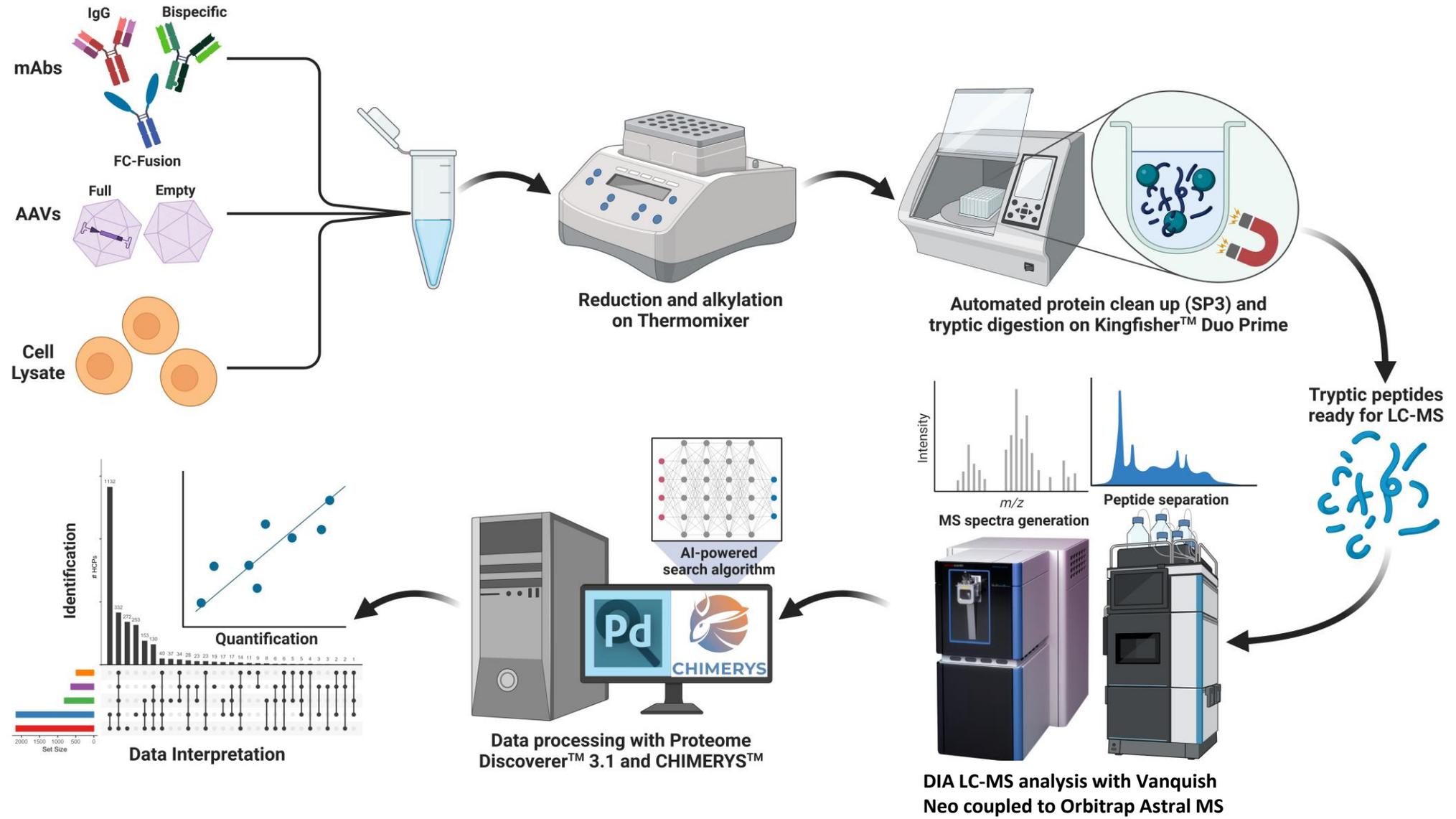
HRAM Scan Rate	Up to 40 Hz
Intrascan dynamic range	>5000 with single microscan
Max Resolution	480,000 at m/z 200
Mass Accuracy	RMS <3 ppm
Max m/z range	Up to m/z 8000 with Biopharma Option



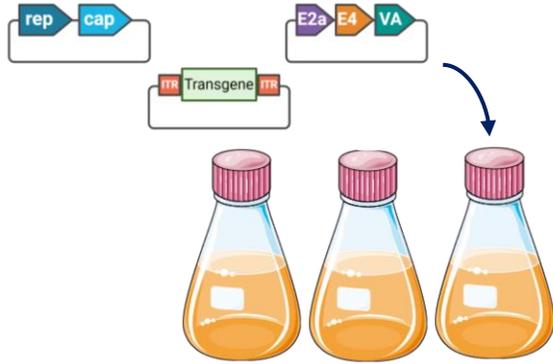
ASTRAL ANALYZER for fast and sensitive high dynamic range HRAM SIM and MS/MS

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

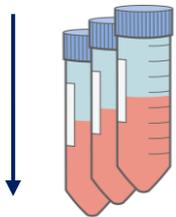
Sample Preparation Workflow



Tracking HCP Clearance using AAVX Affinity Chromatography

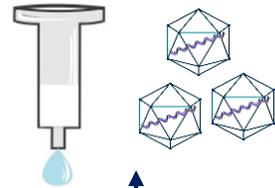


AAV produced in HEK cells using Gibco™ AAV-MAX™ platform and double transfection

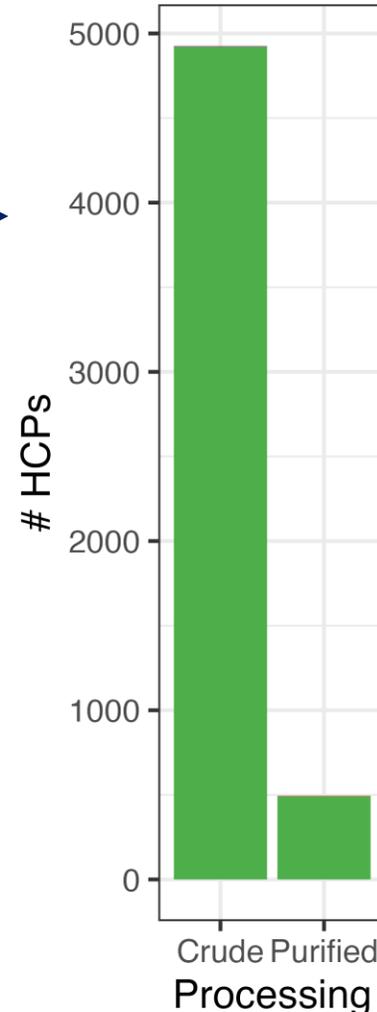


Conditioned media collected, sample retained, remainder purified using POROS™ CaptureSelect™ AAVX Affinity Resin pre-packed column

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



FPLC

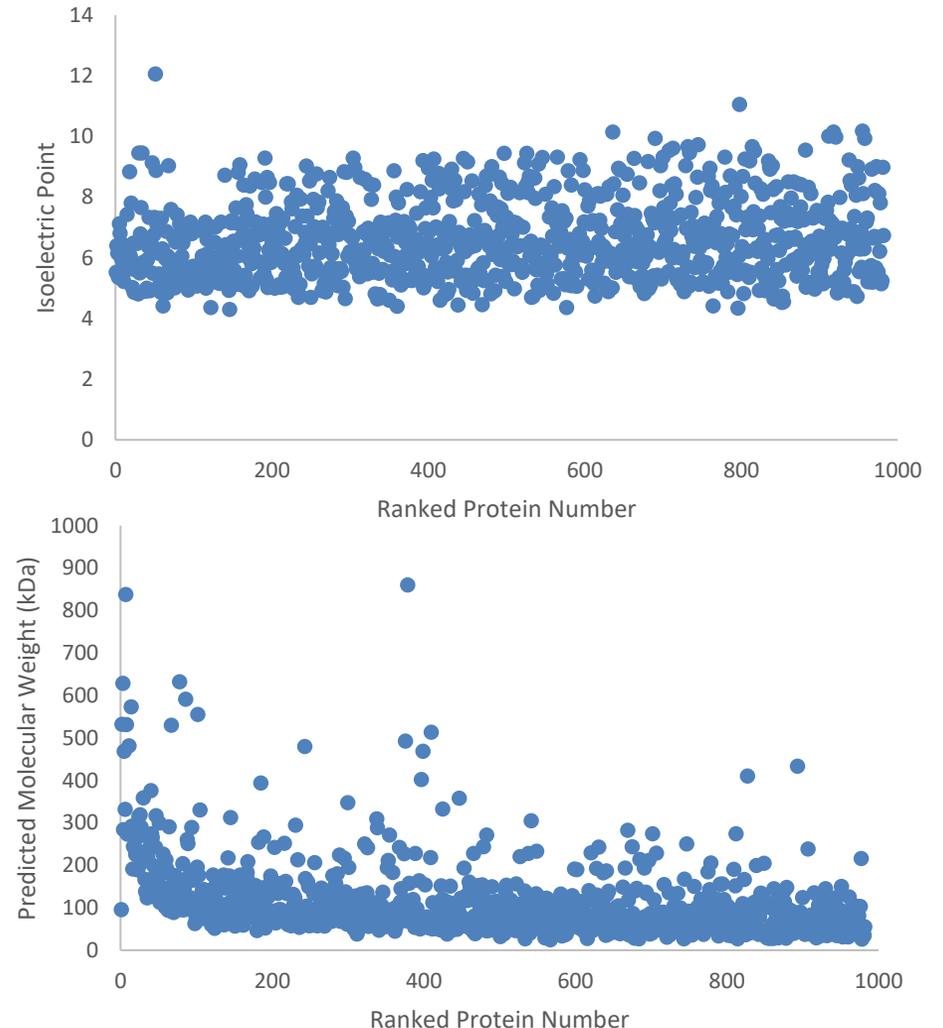


Purification

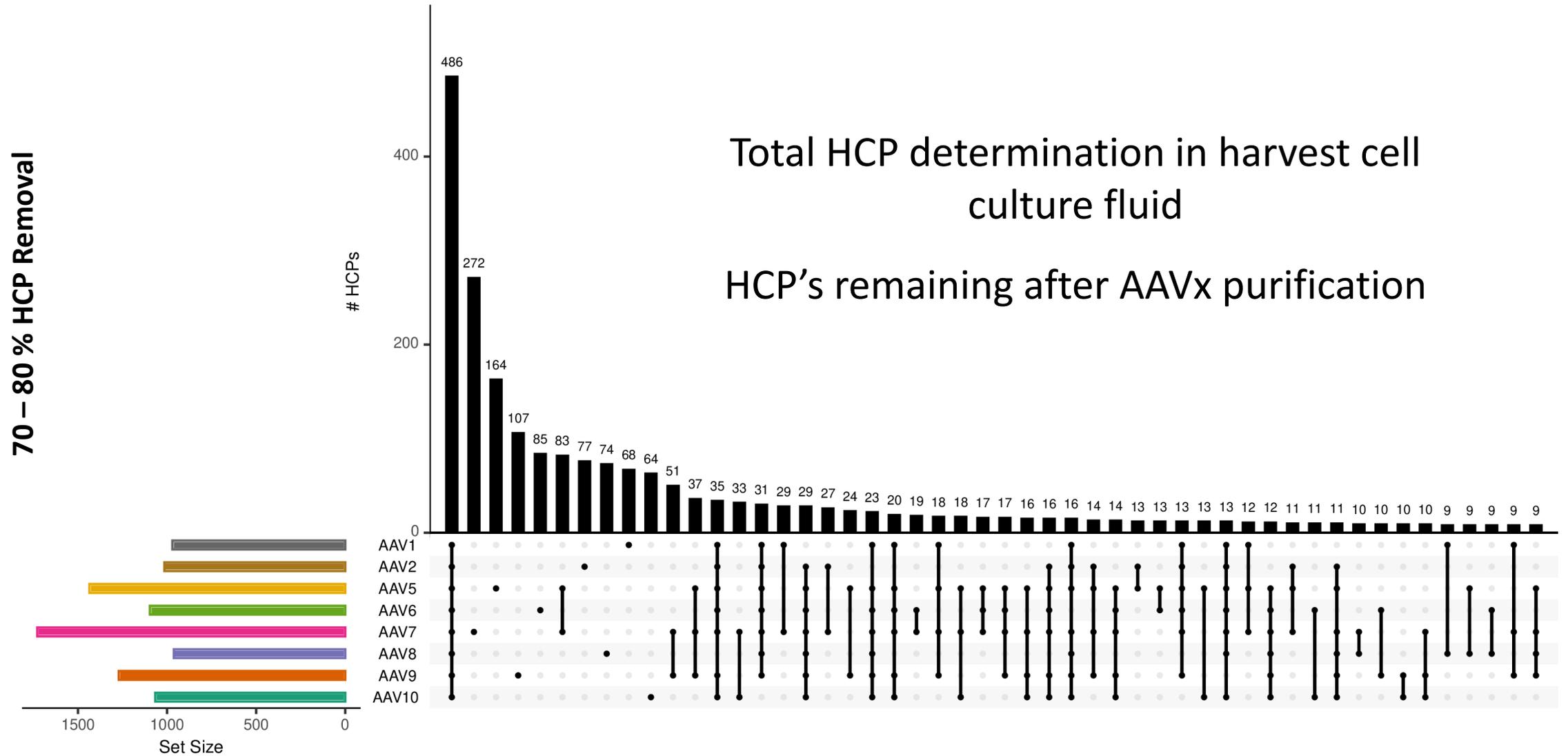
- AAV and AAV Associated Proteins
- Adenovirus C Serpotype 5 Proteins
- Human Proteins

What Remained Associated with Purified CMV-GFP AAV8?

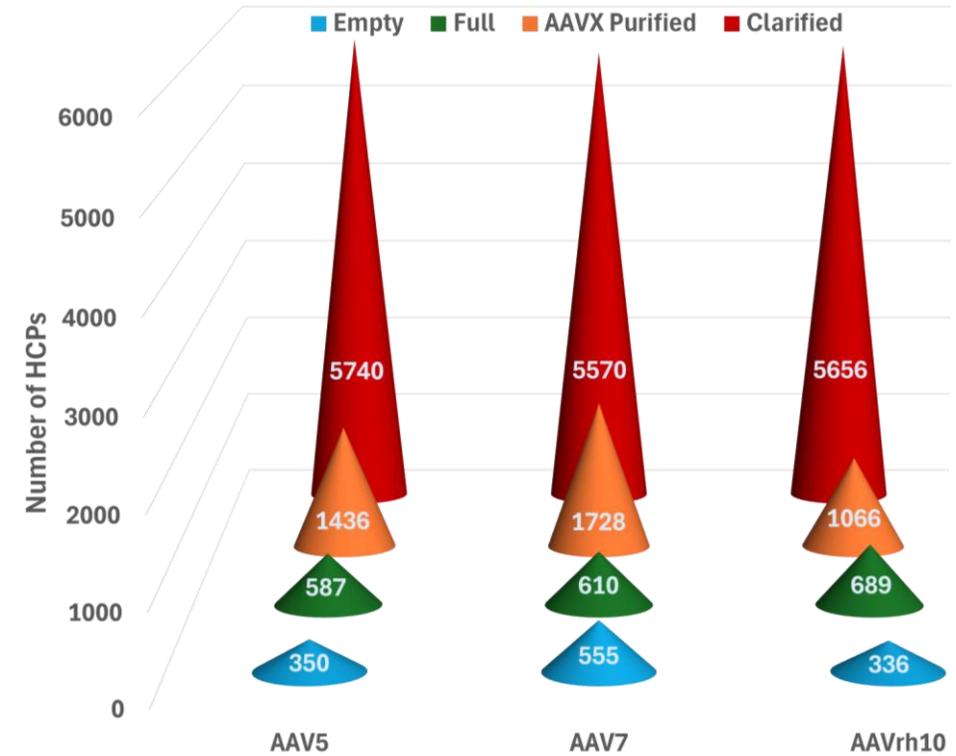
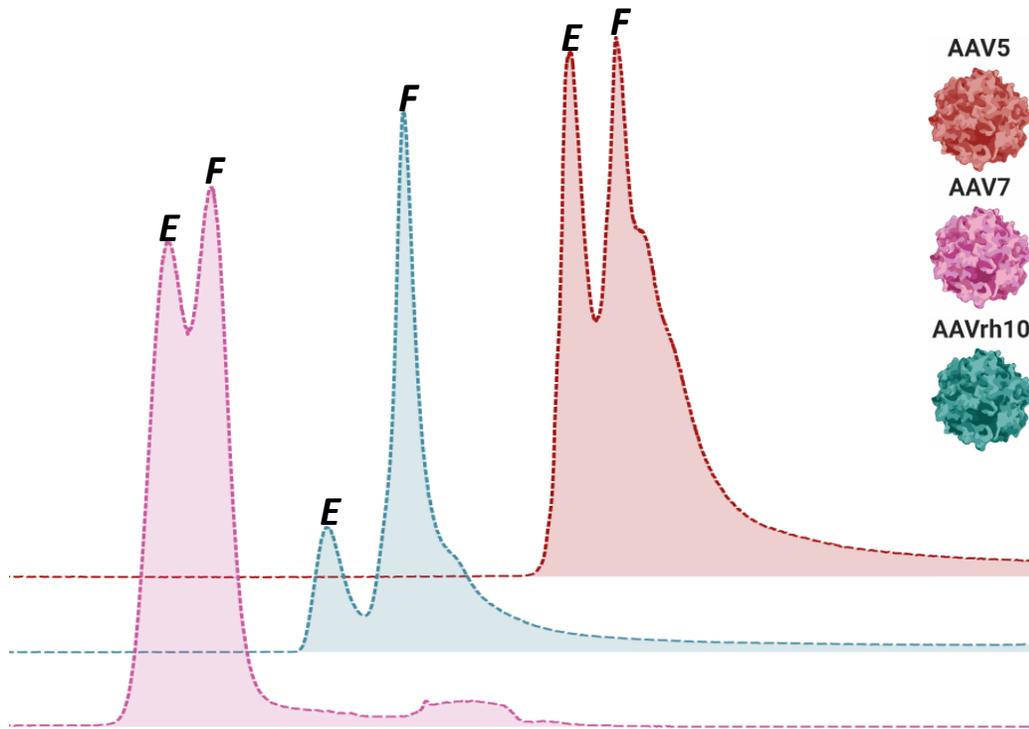
- ❑ AAVX purification resulted in ~80% reduction in the levels of HCPs present in the process stream using a simple bind and elute method.
- ❑ For proteins associated with the retained viral capsids, GO terms relating to binding, in particular protein binding (92.7% of the total set) were enriched. 97.1% 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

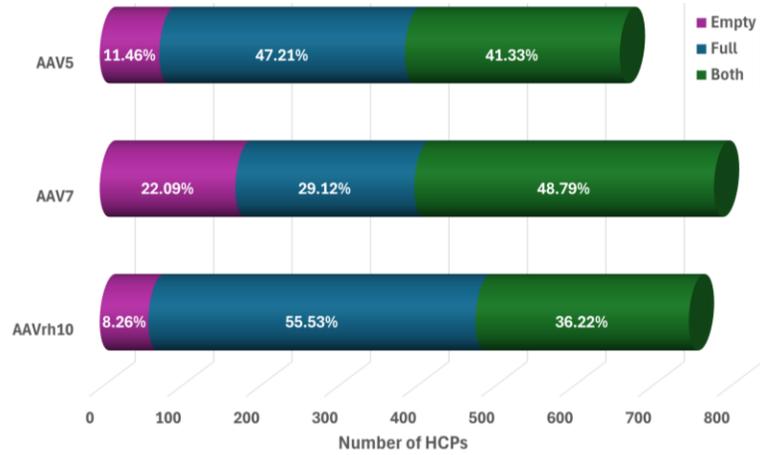


Monitoring Clearance During Downstream Processing

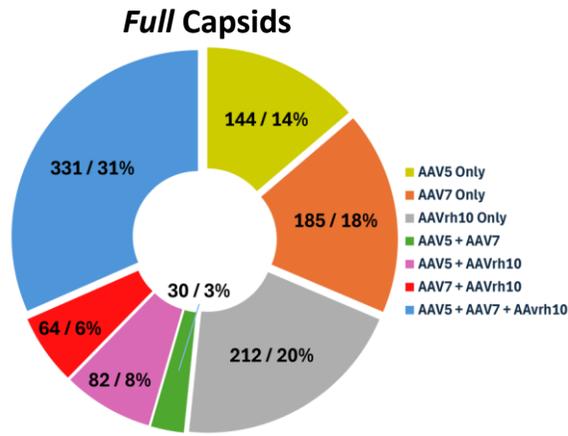
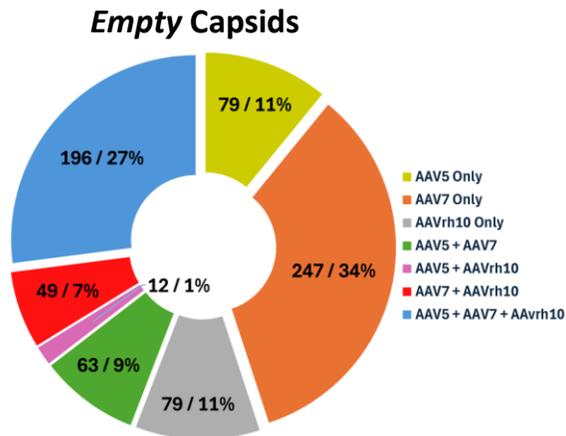


Post AAVx affinity purification, anion exchange separation of empty and full capsids were performed using Poros XQ. Fractions were collected and analysed by LC-MS on Orbitrap Astral to investigate clearance of the HCPs and distribution across the different capsid fill states.

Distribution of HCP across Empty and Full Capsids



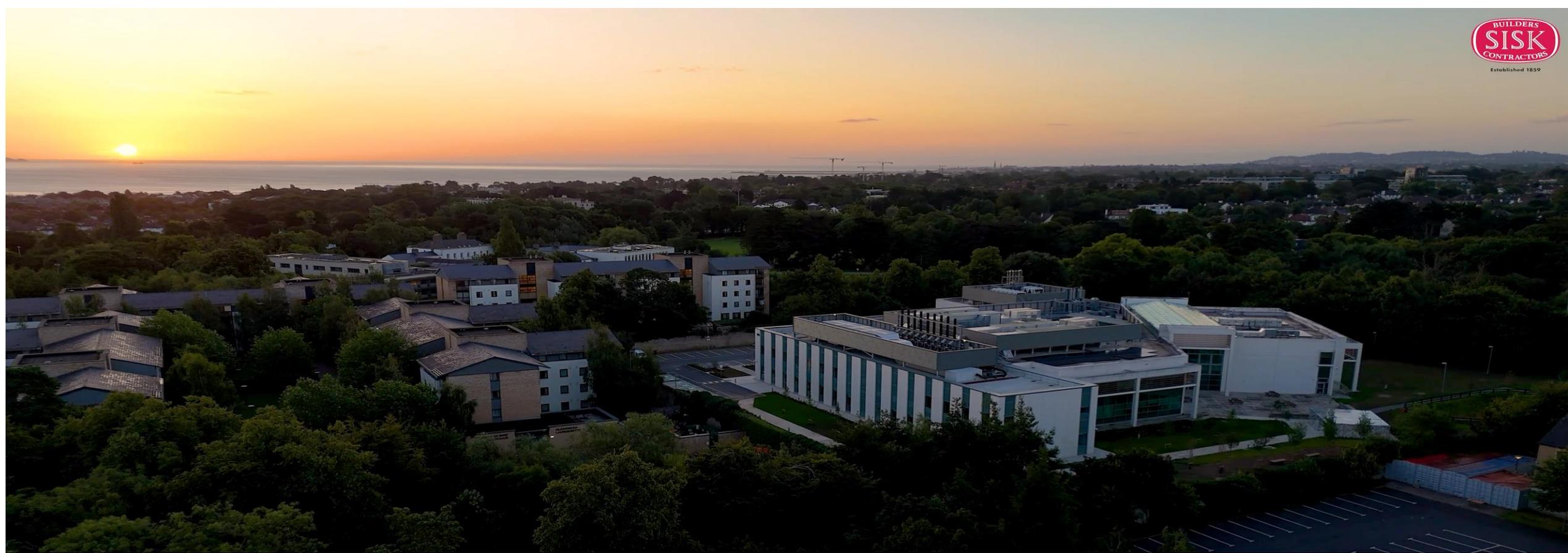
Potentially Harmful HCPs	Clarified			AAVX Purified			Empty			Full		
	AAV5	AAV7	AAVrh10	AAV5	AAV7	AAVrh10	AAV5	AAV7	AAVrh10	AAV5	AAV7	AAVrh10
Heat shock 70 kDa protein 1B	9345.64	9533.52	8595.08	23.49	38.43	15.14	4.68	3.21	2.69	3.34	1.78	2.61
Heat shock protein HSP 90-alpha	2457.25	2392.91	1184.63	7.12	7.92	0.60	0.14	0.25	0.14	0.18	0.54	0.27
Heat shock protein HSP 90-beta	1016.23	1018.65	2064.89	2.81	5.20	2.78	0.19	0.18	0.49	0.17	0.25	0.65
Heat shock cognate 71 kDa protein	736.99	705.87	629.43	3.90	2.07	0.82	0.44	0.36	0.44	0.67	0.36	0.51
60 kDa heat shock protein, mitochondrial	561.73	519.28	544.00	0.66	1.77	0.22	0.07	0.20	0.02	0.15	0.10	0.06
Pyruvate kinase PKM	383.34	381.16	334.20	3.41	6.72	2.35	0.26	0.51	0.51	0.88	0.92	1.41
DNA-binding protein	185.63	242.56	258.81	0.03	0.20	0.07		0.06				
Histone H1.4	116.54	99.24	100.20									
Histone H4	75.59	63.65	67.62	3.51	4.46	1.43	0.29	0.24	0.23	0.38	0.46	0.48
Protein disulfide-isomerase	64.42	47.73	70.53	0.09	0.14	0.01		0.03		0.02	0.09	0.05
E1B 55 kDa protein	63.24	58.20	47.12									
Annexin A2	57.37	53.66	51.90	3.66	0.38	0.80	0.70	1.56	0.92	2.08	1.18	2.23
Peroxioredoxin-2	37.52	33.10	37.38	2.96	0.30	0.27	0.63	1.51	0.25	2.36	1.56	2.28
E1B protein, small T-antigen	28.93	26.99	22.42	0.02	0.03							



- As expected, ability to separate empty and full capsids effected the ability to differentiate HCP loads, however some specificity was observed.
- Similarly, specificity was observed for the serotypes analysed.
- ‘Problematic HCPs’ were investigated in the resulting LC-MS data to evaluate their clearance, as shown in the heatmap, the majority were cleared by AAVx affinity chromatography.

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:

Josh Smith, Corentin Beaumal, Sara Carillo, Aaron Richardson, Felipe Guapo, Colin Clarke, Florian Füssl, Lisa Strasser, Silvia Millán-Martín

Thermo Fisher Scientific:

Eugen Damoc, Anna Pashkova, Kristina Srzentic, Tabiwang N. Arrey, Kai Scheffler, Kelly Broster, David M. Horn, Min Du, Steve G. Milian, Richard O. Snyder

908 Devices:

Erin Redman



CONCEPT

More info: concept-nibrt.ie