

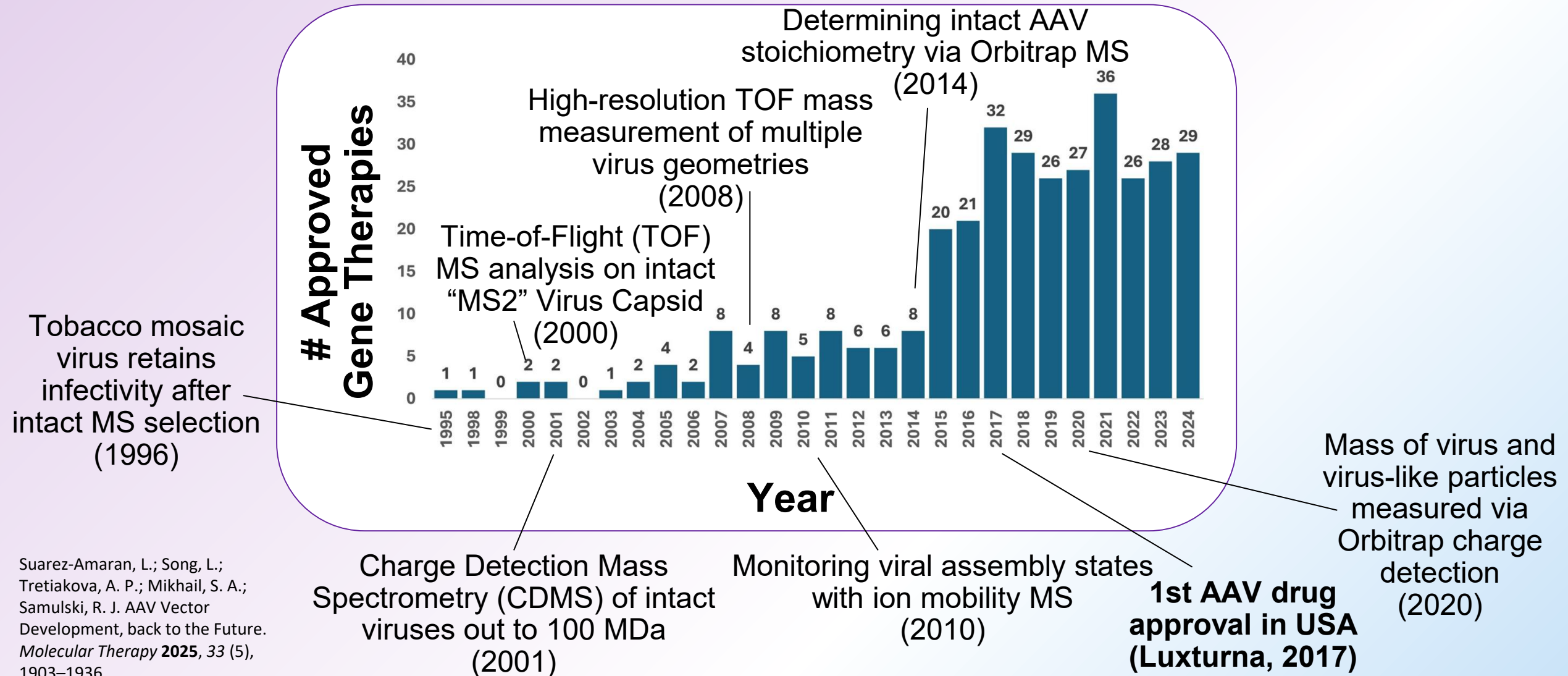
Monitoring Intact AAV and AAV Building Blocks Through Individual Ion Mass Spectrometry

**Jared Kafader, Samuel Janisse, Neil Kelleher, and
John McGee**



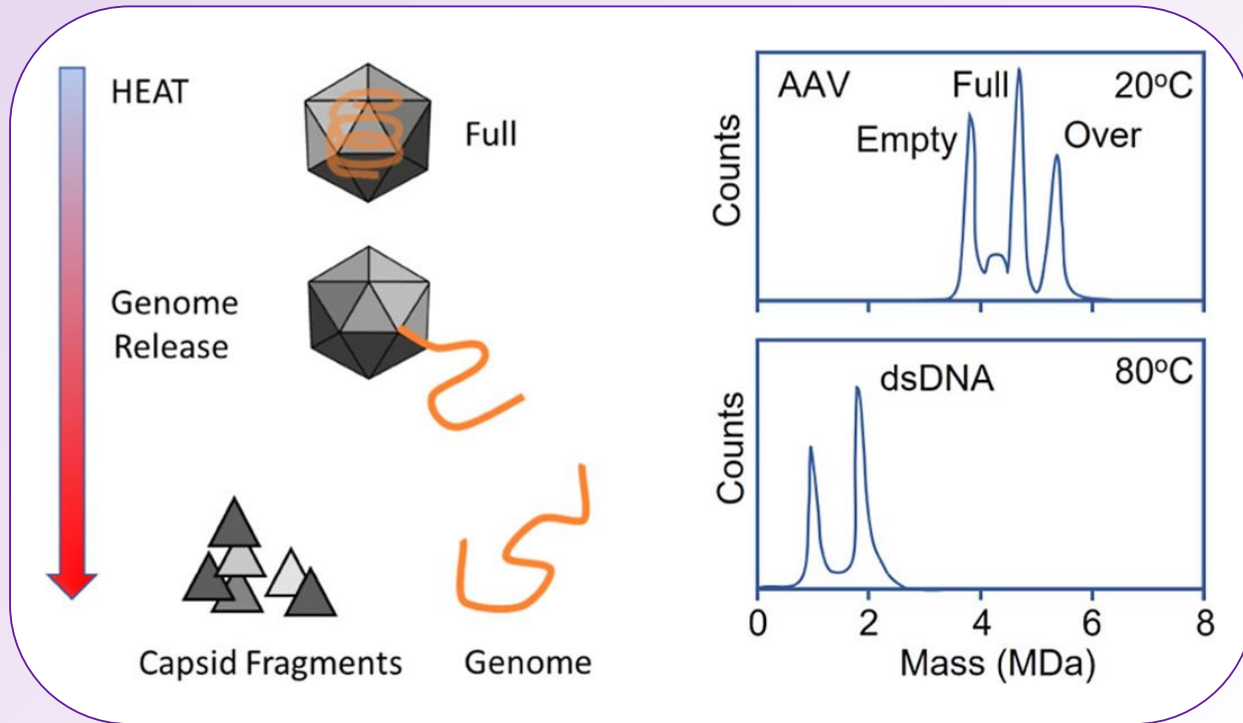
jack@immpo.net

Mass Spectrometry And Viruses



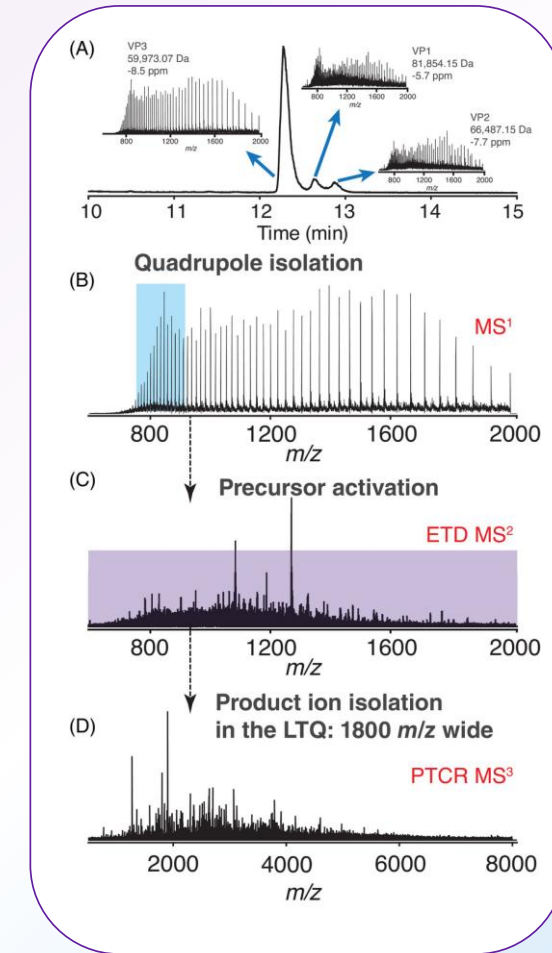
Mass Spectrometry Methods For AAVs Are Still Evolving

Intact & Cargo Analysis



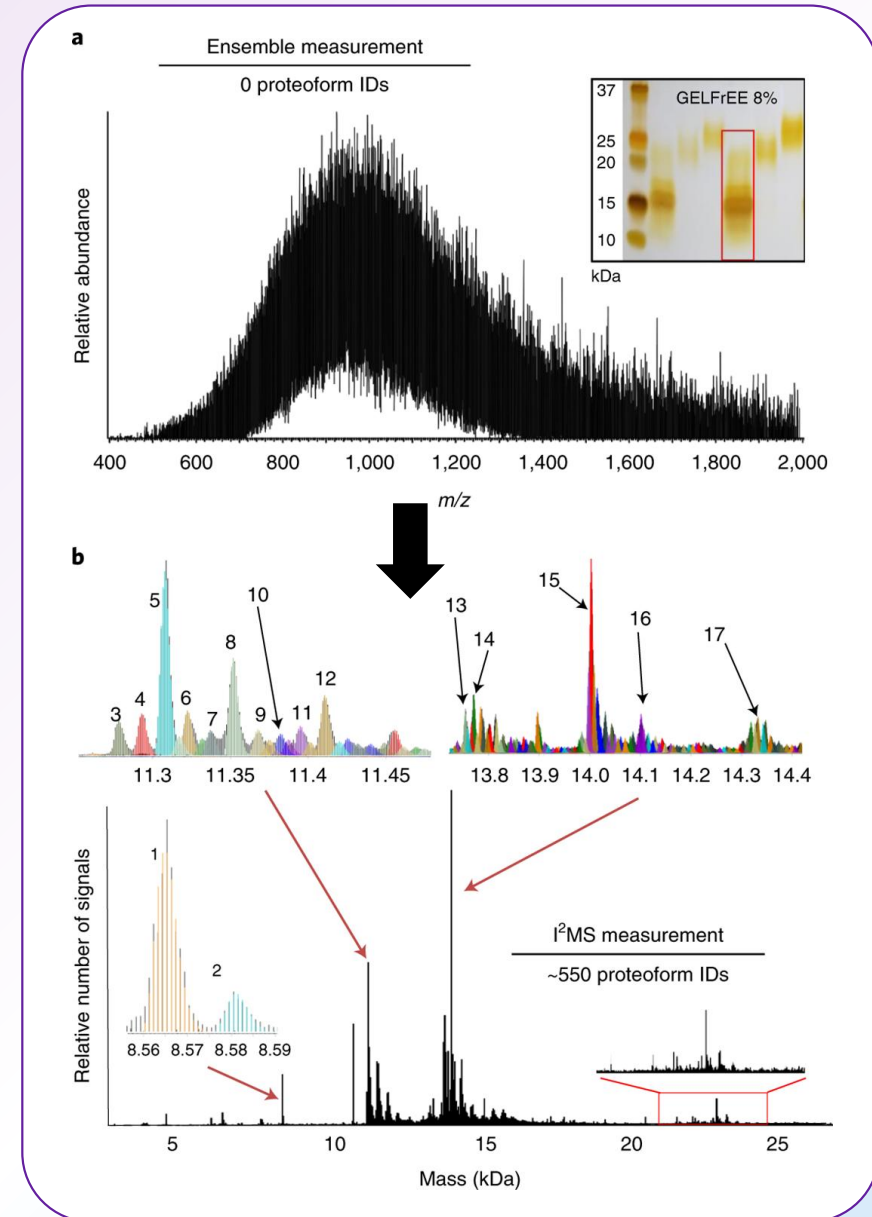
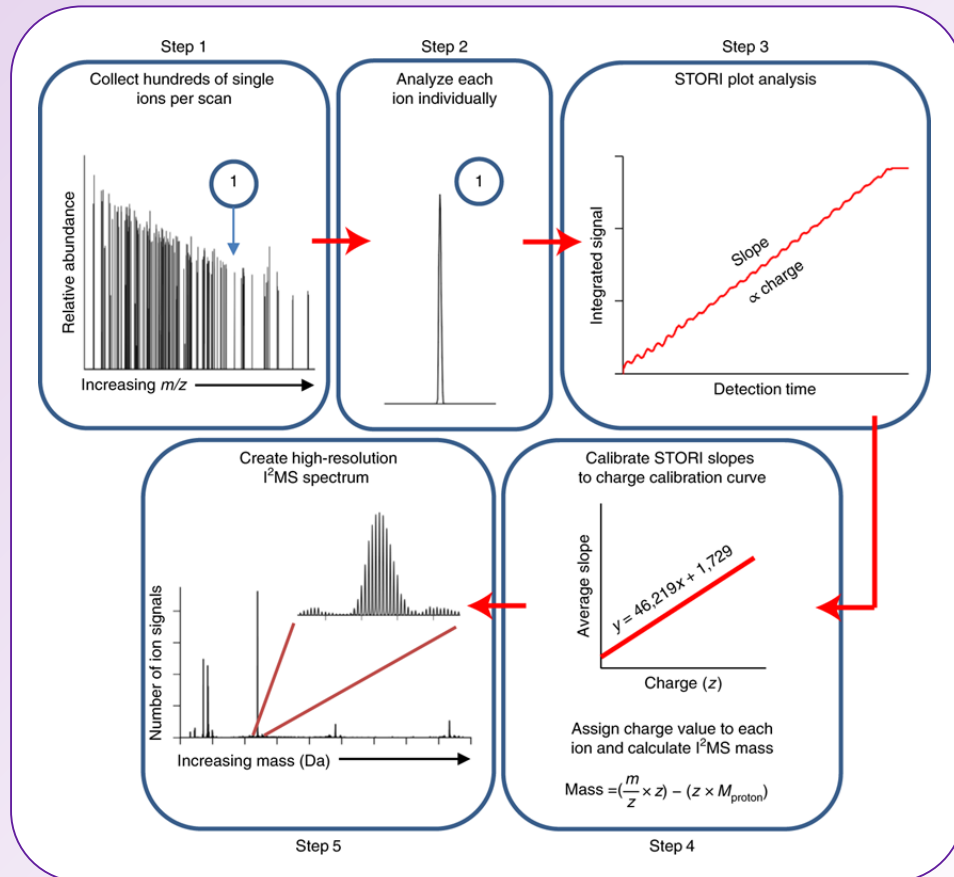
Barnes, L. F. et al. Analysis of AAV-Extracted DNA by Charge Detection Mass Spectrometry Reveals Genome Truncations. *Analytical Chemistry* **2023**, 95 (9), 4310-4316.

Component Analysis



Kline, J. T.. et al. Top-down Mass Spectrometry Analysis of Capsid Proteins of Recombinant Adeno-Associated Virus Using Multiple Ion Activations and Proton Transfer Charge Reduction. *PROTEOMICS* **2024**.

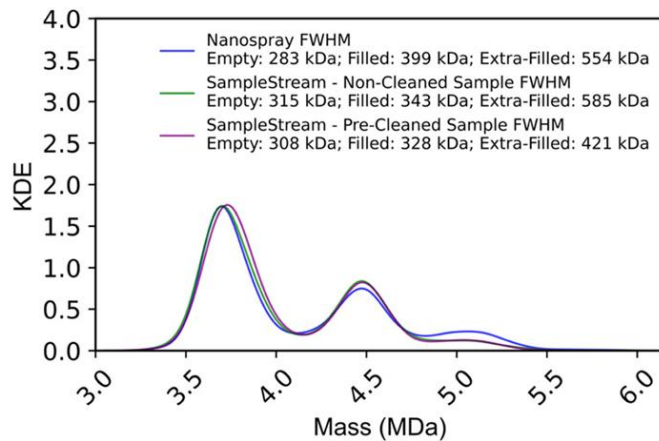
Individual Ion Mass Spectrometry (I²MS)



Kafader J. O. et al. Multiplexed Mass Spectrometry of Individual Ions Improves Measurement of Proteoforms and Their Complexes. *Nature Methods* **2020**, 17 (4), 391-394.

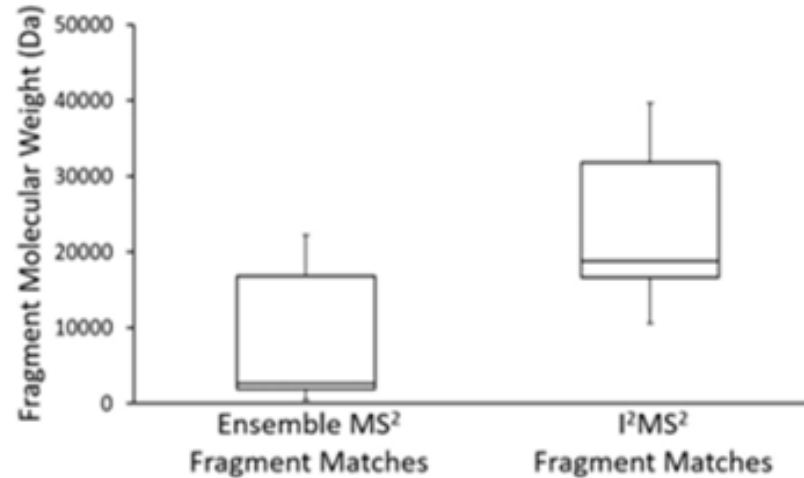
I2MS Can Analyze...

AAVs



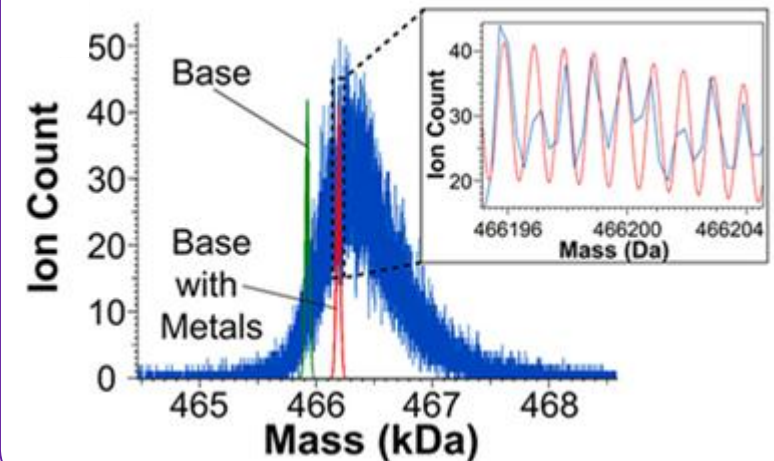
Janisse, S. E. et al. A Robust and Automated Platform for Charge Detection Mass Spectrometry of Megadalton Biotherapeutics. *Analytical Chemistry* **2025**, 97 (8), 4549-4555.

AND large fragment ions



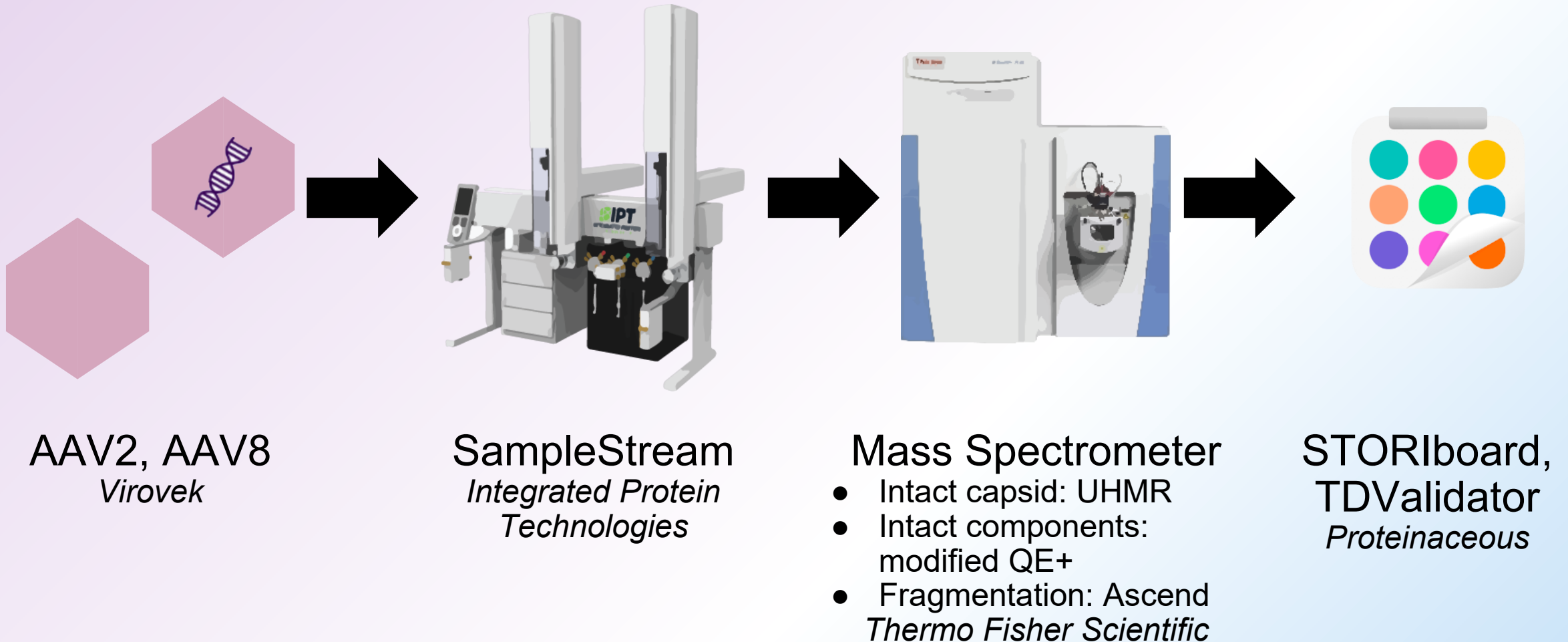
Kafader, J. O. et al. Individual Ion Mass Spectrometry Enhances the Sensitivity and Sequence Coverage of Top-down Mass Spectrometry. *Journal of Proteome Research* **2020**, 19 (3), 1346-1350.

precisely, across a high potential molecular weight range



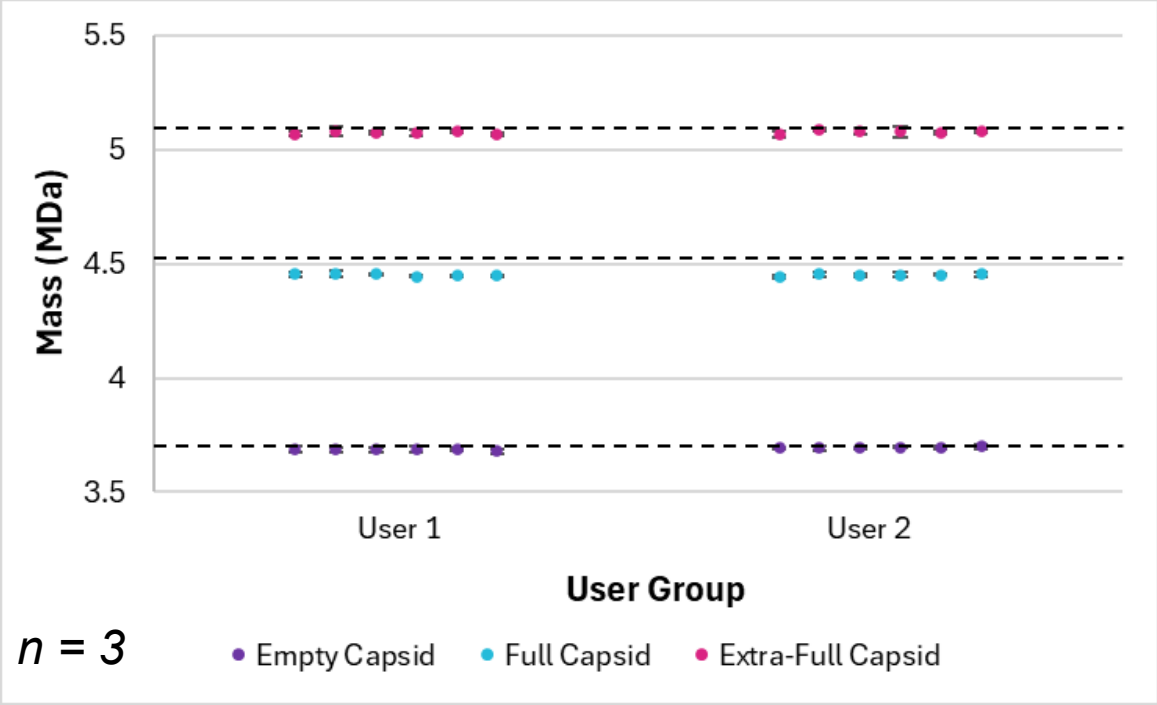
McGee, J.P. et al. Isotopic Resolution of Protein Complexes up to 466 KDa Using Individual Ion Mass Spectrometry. *Analytical Chemistry* **2020**, 93 (5), 2723-2727.

The Platform



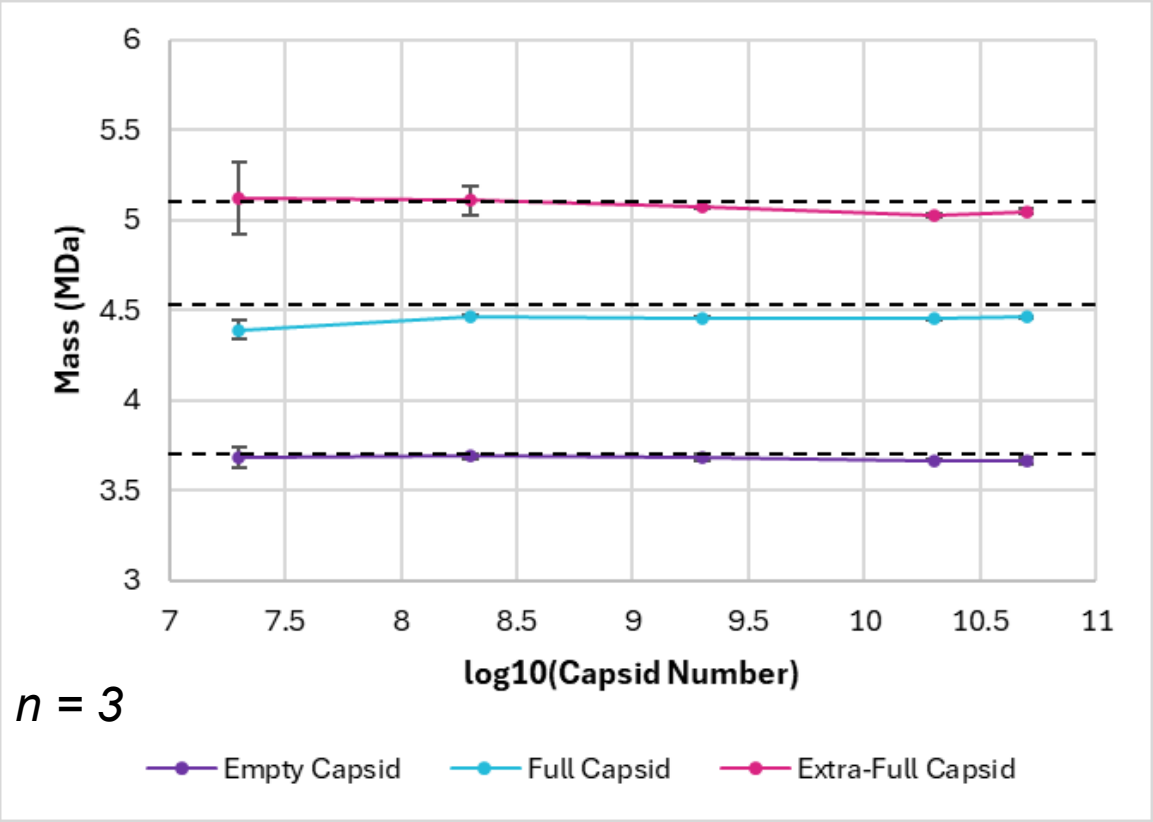
I: The Intact Capsid

Accuracy, Repeatability, and Intermediate Precision: Mass



	Empty Mass (% Error), MDa	Full Mass (% Error), MDa	Extra-Full Mass (% Error), MDa
Analyst 1	3.69 ±0.01 (0.3%)	4.45 ±0.01 (1.8%)	5.07 ±0.01 (0.6%)
Analyst 2	3.70 ±0.01 (0%)	4.45 ±0.01 (1.8%)	5.08 ±0.01 (0.4%)
Theoretical	3.70	4.53	5.10

Linearity: Mass

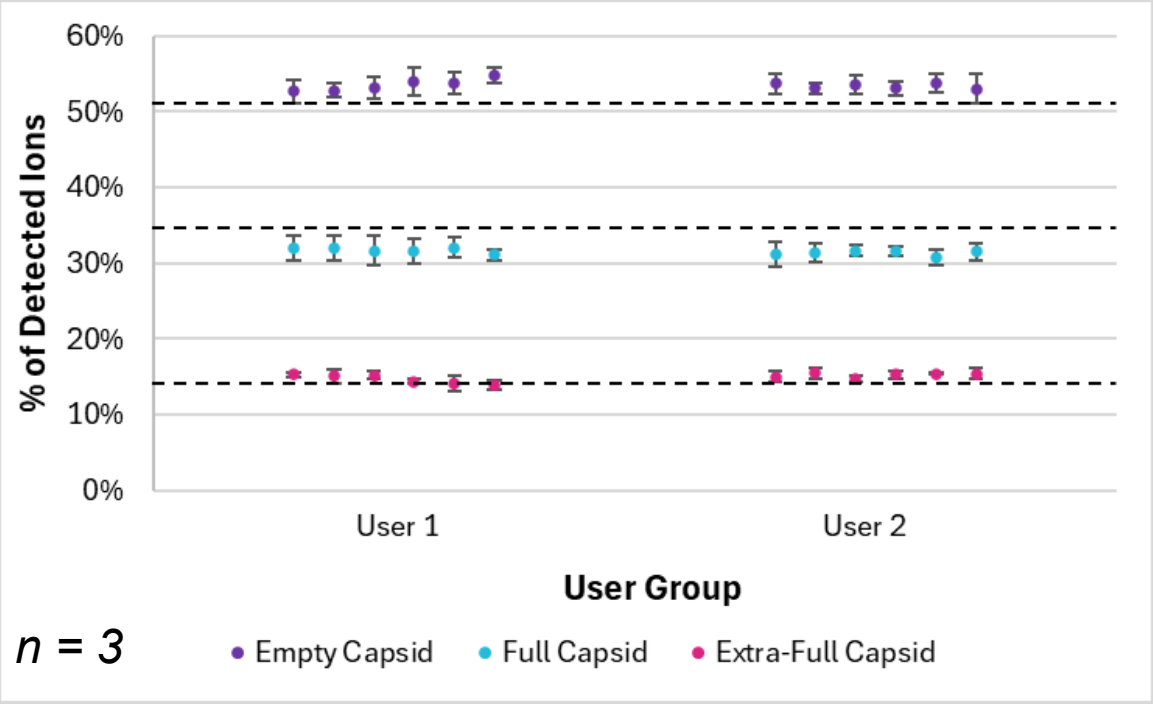


Linear Regression t-Test
 H_0 : Slope = 0, $\alpha = 0.05$

	p-value	Conclusion
Extra-Full Capsid	0.12	Fail to reject H_0
Full Capsid	0.20	Fail to reject H_0
Empty Capsid	0.11	Fail to reject H_0

Recommended Range: 2e8 - 5e10

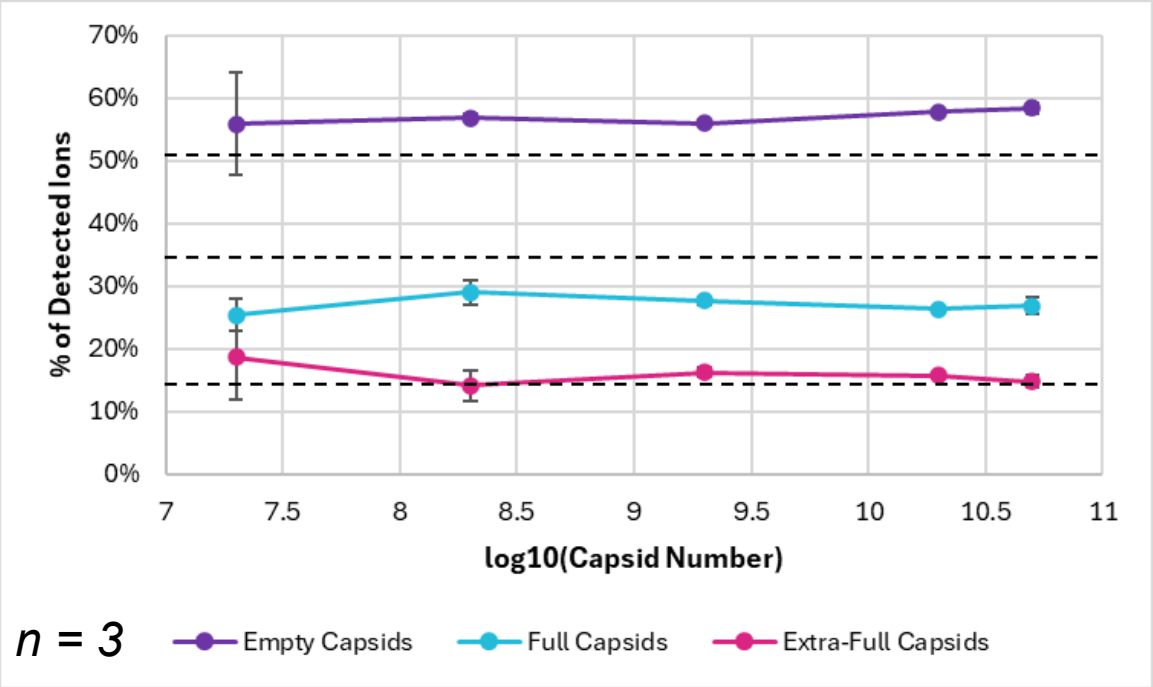
Accuracy, Repeatability, and Intermediate Precision: Ratios



	Empty Amount (% Error)	Amount (% Error)	Extra-Full (% Error)
Analyst 1	53.5% ±1.4% (4.7%)	31.8% ±1.3% (8.4%)	14.7% ±0.8% (3.5%)
Analyst 2	53.4% ±1.1% (4.5%)	31.4% ±1.0% (9.5%)	15.3% ±0.6% (7.7%)
Theoretical*	51.1%	34.7%	14.2%

**50:50 mixture of empty and full product, where the full product was individually characterized as 2.2% empty and 28.5% extra-full*

Linearity: Ratios



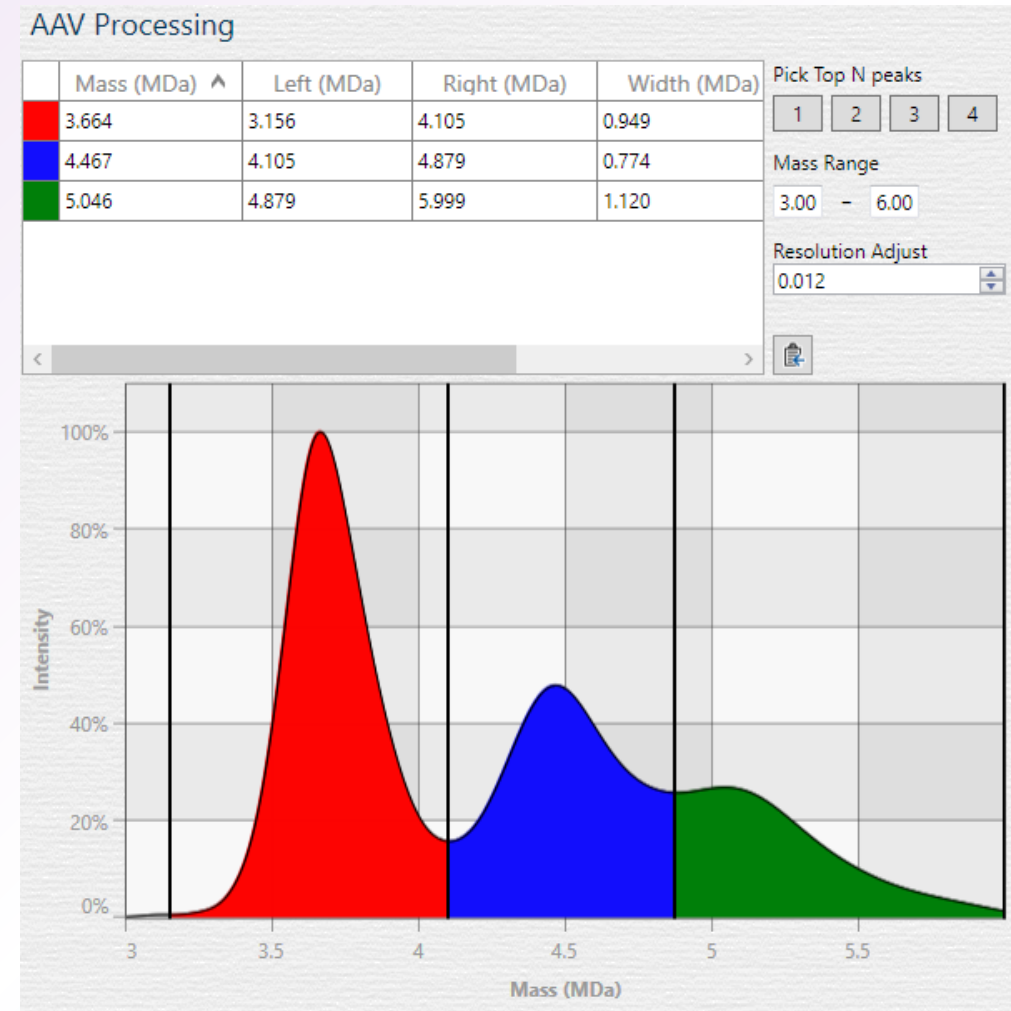
Linear Regression t-Test
 H_0 : Slope = 0, $\alpha = 0.05$

	p-value	Conclusion
Extra-Full Capsid	0.33	Fail to reject H_0
Full Capsid	0.94	Fail to reject H_0
Empty Capsid	0.08	Fail to reject H_0

Recommended Range: 2e8 - 5e10

I: The Intact Capsid

- Mass readings
 - Highly accurate (Error <2%)
 - Stable across different users
 - Stable across ~2 orders of magnitude input
- Ratio readings
 - Moderately accurate with stable bias (Error <10%)
 - Unclear if due to product-related fluctuations
 - Stable across different users
 - Stable across ~2 orders of magnitude input
- Throughput
 - 10-70 ions/second
 - >70 samples/day

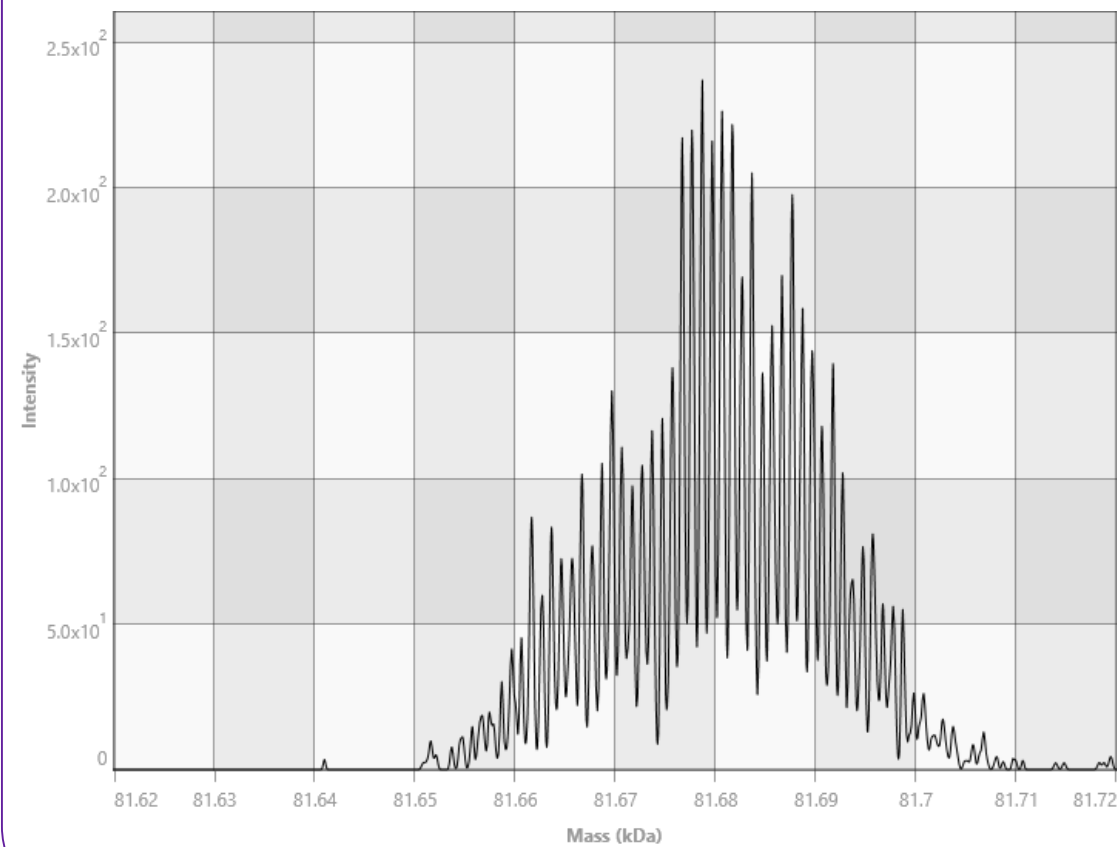
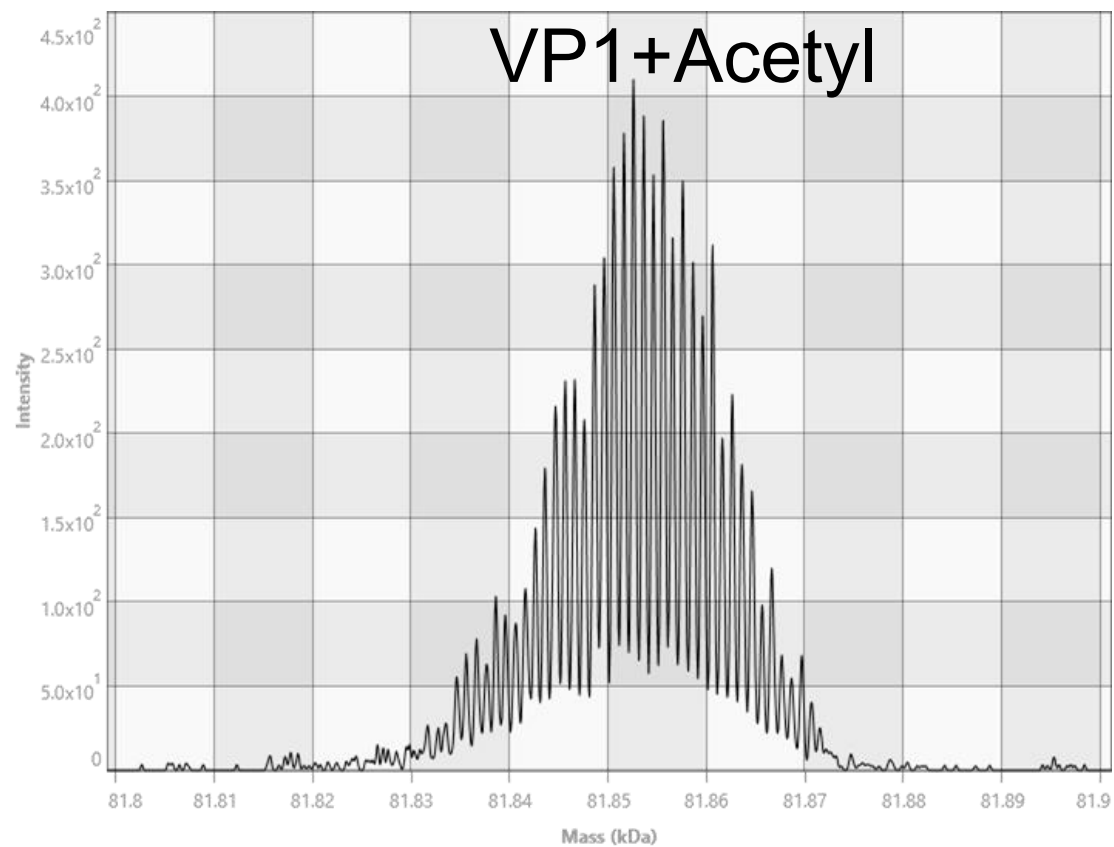


II: The Intact Components

VP1

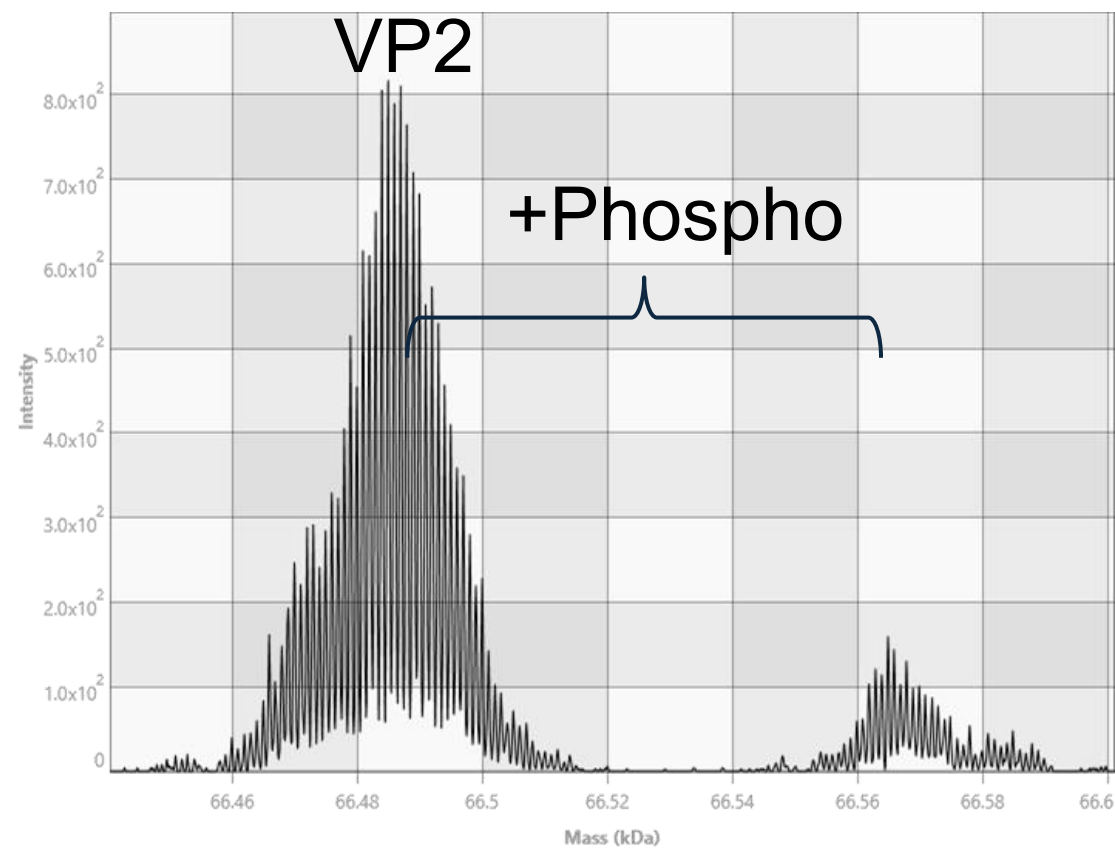
AAV2

AAV8

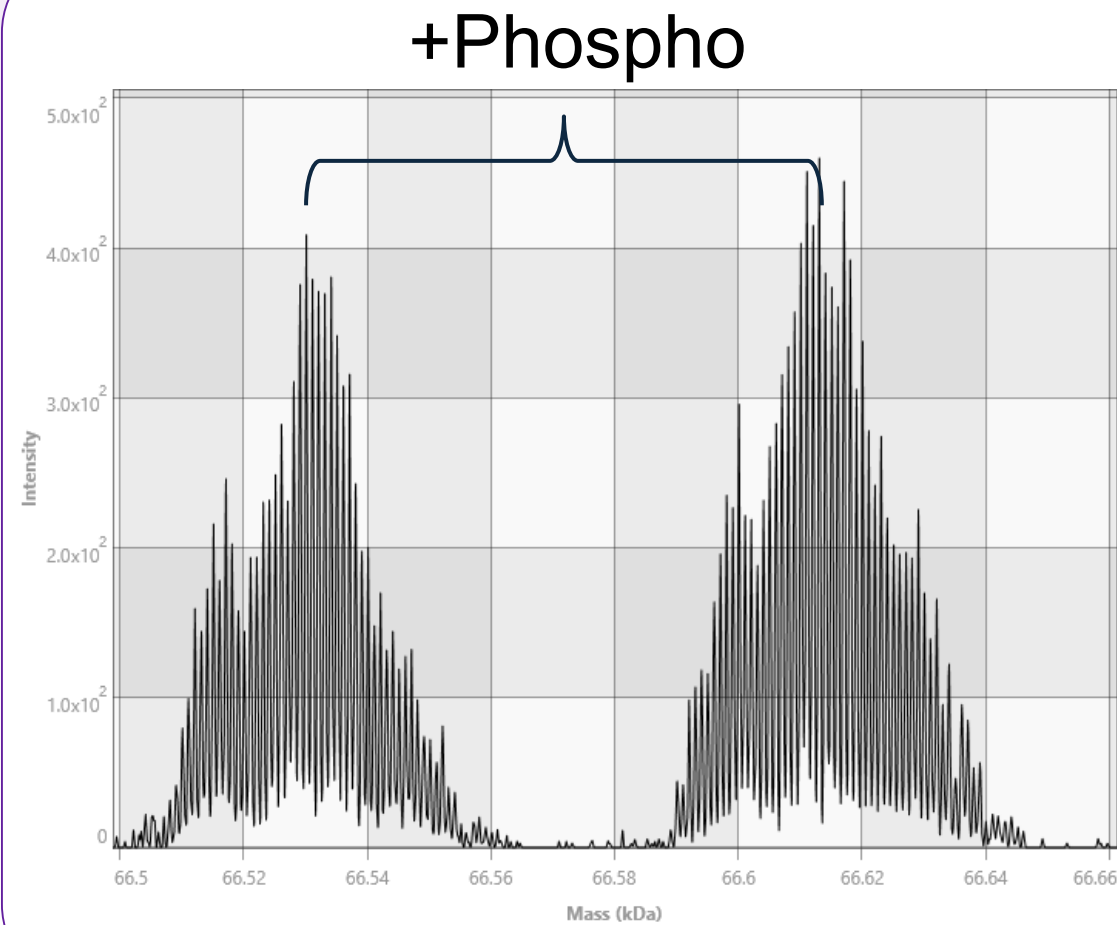


VP2

AAV2

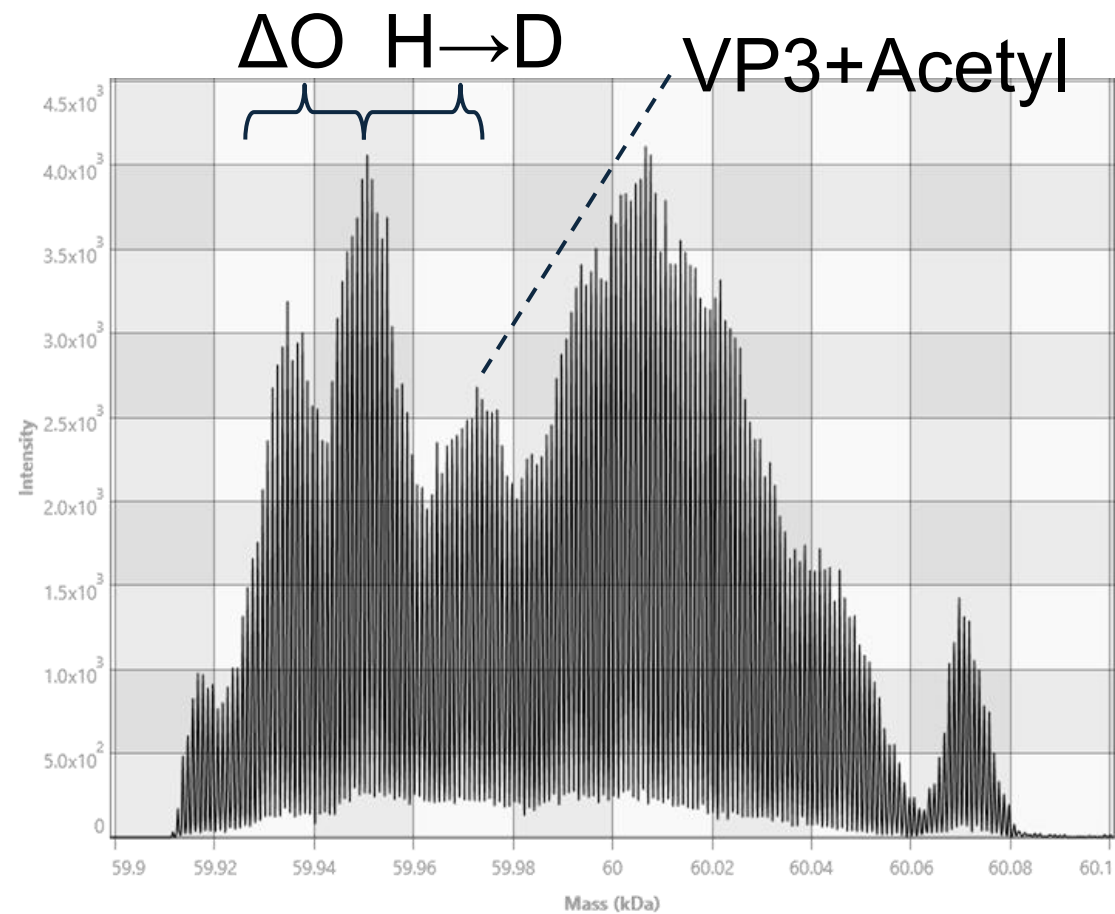


AAV8

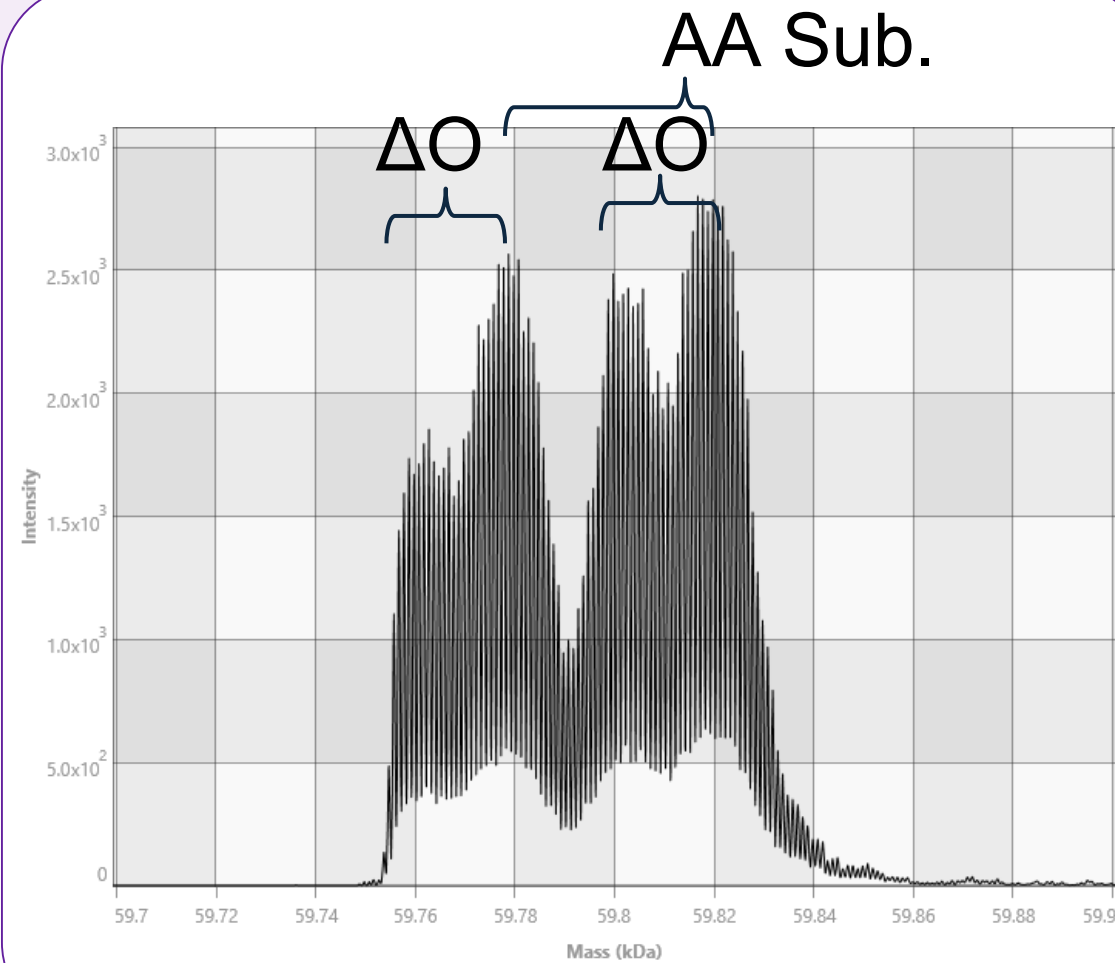


VP3

AAV2



AAV8



II: The Intact Components

- Various proteoforms across the VPs and isotypes
- Likely phosphorylation for VP2
- Likely multiple sequences for VP3

III: Fragmentation

VP1 (AAV2)

I2MS

PTCR MS³*

| a/x ions | b/y ions | c/z ions

```

25  A A D G Y L P D W L E D T L S E G I R Q W W K L K
26  P G P P P P K P A E R H K D D S R G L V L P G Y K
51  Y L G P F N G L D K G E P V N E A D A A A L E H D
76  K A Y D R Q L D S G D N P Y L K Y N H A D A E F Q
101 E R L K E D T S F G G N L G R A V F Q A K K R V L
126 E P L G L V E E P V K T A P G K K R P V E H S P V
151 E P D S S S G T G K A G Q Q P A R K R L N F G Q T
176 G D A D S V P D P Q P L G Q P P A A P S G L G T N
201 T M A T G S G A P M A D N N E G A D G V G N S S G
226 N W H C D S T W M G D R V I T T S T R T W A L P T
251 Y N N H L Y K Q I S S Q S G A S N D N H Y F G Y S
276 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
301 N N W G F R P K R L N F K L F N I Q V K E V T Q N
326 D G T T T I A N N L T S T V Q V F T D S E Y Q L P
351 Y V L G S A H Q G C L P P F P A D V F M V P Q Y G
376 Y L T L N N G S Q A V G R S S F Y C L E Y F P S Q
401 M L R T G N N F T F S Y T F E D V P F H S S Y A H
426 S Q S L D R L M N P L I D Q Y L L Y Y L S R T N T P
451 S G T T T Q S R L Q F S Q A G A S D I R D Q S R N
476 W L P G P C Y R Q Q R V S K T S A D N N N S E Y S
501 W T G A T K Y H L N G R D S L V N P G P A M A S H
526 K D D E E K F F P Q S G V L I F G K Q G S E K T N
551 V D I E K V M I T D E E E I R T T N P V A T E Q Y
576 G S V S T N L Q R G N R Q A A T A D V N T Q G V L
601 P G M V W Q D R D V Y L Q G P I W A K I P H T D G
626 H F H P S P L M G G F G L K H P P P Q I L I K N T
651 P V P A N P S T T F S A A K F A S F I T Q Y S T G
676 Q V S V E I E W E L Q K E N S K R W N P E I Q Y T
701 S N Y N K S V N V D F T V D T N G V Y S E P R P I
726 G T R Y L T R N L C
    
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Coverage: 15.3%

```

25  A A D G Y L P D W L E D T L S E G I R Q W W K L K
26  P G P P P P K P A E R H K D D S R G L V L P G Y K
51  Y L G P F N G L D K G E P V N E A D A A A L E H D
76  K A Y D R Q L D S G D N P Y L K Y N H A D A E F Q
101 E R L K E D T S F G G N L G R A V F Q A K K R V L
126 E P L G L V E E P V K T A P G K K R P V E H S P V
151 E P D S S S G T G K A G Q Q P A R K R L N F G Q T
176 G D A D S V P D P Q P L G Q P P A A P S G L G T N
201 T M A T G S G A P M A D N N E G A D G V G N S S G
226 N W H C D S T W M G D R V I T T S T R T W A L P T
251 Y N N H L Y K Q I S S Q S G A S N D N H Y F G Y S
276 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
301 N N W G F R P K R L N F K L F N I Q V K E V T Q N
326 D G T T T I A N N L T S T V Q V F T D S E Y Q L P
351 Y V L G S A H Q G C L P P F P A D V F M V P Q Y G
376 Y L T L N N G S Q A V G R S S F Y C L E Y F P S Q
401 M L R T G N N F T F S Y T F E D V P F H S S Y A H
426 S Q S L D R L M N P L I D Q Y L Y Y L S R T N T P
451 S G T T T Q S R L Q F S Q A G A S D I R D Q S R N
476 W L P G P C Y R Q Q R V S K T S A D N N N S E Y S
501 W T G A T K Y H L N G R D S L V N P G P A M A S H
526 K D D E E K F F P Q S G V L I F G K Q G S E K T N
551 V D I E K V M I T D E E E I R T T N P V A T E Q Y
576 G S V S T N L Q R G N R Q A A T A D V N T Q G V L
601 P G M V W Q D R D V Y L Q G P I W A K I P H T D G
626 H F H P S P L M G G F G L K H P P P Q I L I K N T
651 P V P A N P S T T F S A A K F A S F I T Q Y S T G
676 Q V S V E I E W E L Q K E N S K R W N P E I Q Y T
701 S N Y N K S V N V D F T V D T N G V Y S E P R P I
726 G T R Y L T R N L C
    
```

Coverage: 28.4%
ETD-Only: 18.3%

Kline, J. T. et al. Top-down mass spectrometry analysis of capsid proteins of recombinant adeno-associated virus using multiple ion activations and proton transfer charge reduction. *Proteomics* **2024**.

VP2 (AAV2)

I2MS

PTCR MS³*

| a/x ions

| b/y ions

| c/z ions

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

Coverage: 20.6%

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

Coverage: 28.1%

ETD-Only: 18.9%

Kline, J. T. et al. Top-down mass spectrometry analysis of capsid proteins of recombinant adeno-associated virus using multiple ion activations and proton transfer charge reduction. *Proteomics* **2024**.

VP3 (AAV2)

I2MS

PTCR MS³*

 a/x ions

 b/y ions

 c/z ions

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

Coverage: 37.7%

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

Coverage: 39.1%

ETD-Only: 31.6%

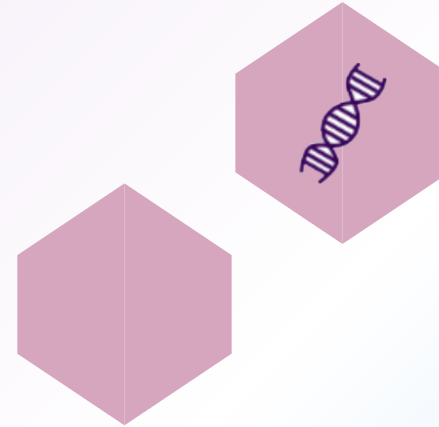
Kline, J. T. et al. Top-down mass spectrometry analysis of capsid proteins of recombinant adeno-associated virus using multiple ion activations and proton transfer charge reduction. *Proteomics* **2024**.

III: Fragmentation

- Larger fragment ions than provided by PTCLR MS³
 - Greater ability to localize internal post-translational modifications
- Coverage comparable to PTCLR-ETD MS³
- Lack of N-terminal fragment ions

Key Takeaways

- The tools for AAV characterization are rapidly maturing
- I2MS can address several angles of AAV product characterization
 - Intact analysis
 - Diverse proteoform component measurement
 - Proteoform fragmentation
- A wholistic I2MS platform for AAVs can hugely impact accessibility of this space



Acknowledgements



Neil Kelleher, PhD



Jared Kafader, PhD



Samuel Janisse, PhD



Choon Aun Quek



Vincent White



Lola Lauer

Monitoring Intact AAV and AAV Building Blocks Through Individual Ion Mass Spectrometry

**Jared Kafader, Samuel Janisse, Neil Kelleher, and
John McGee**



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Timeline References

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