Product Structural Characterization for AAV-based Gene Therapy Development

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Adeno-associated Virus (AAV) for Gene Therapy

Gene therapy is the therapeutic delivery of nucleic acid into a patient as a drug to treat disease.

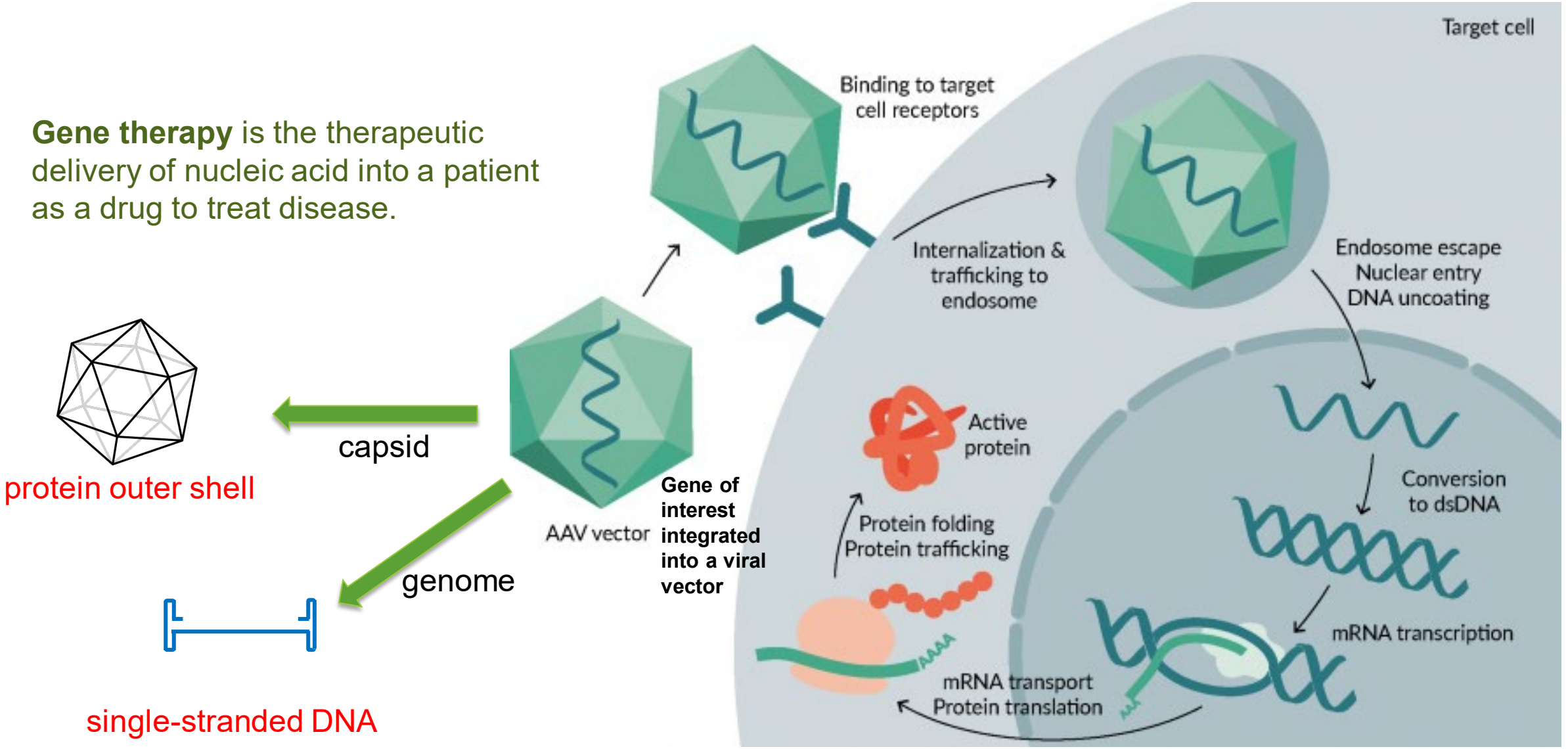
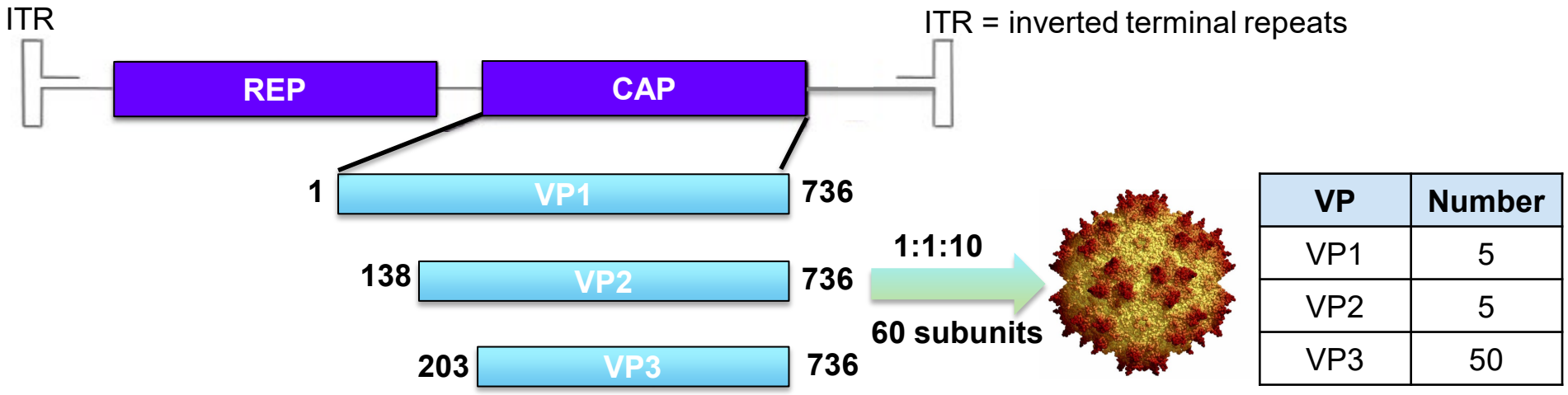


Figure adapted from: Li, *et al.* 2019, Cell & Gene Therapy Insights 2019; 5(4), 537–547

Wild Type AAV Structural Characteristics and Quality Attributes



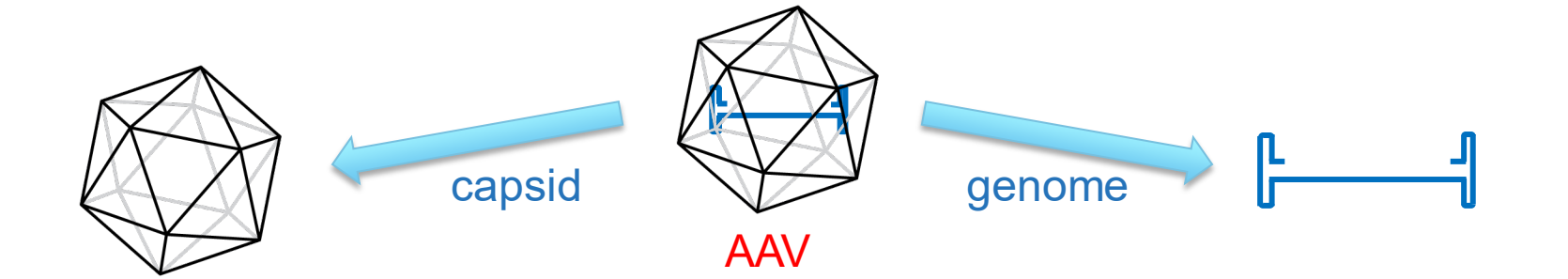
VP	Number
VP1	5
VP2	5
VP3	50

Capsid Proteins - Viral Proteins (VPs)

Adeno-Associated Virus
3.9 MegaDaltons (empty capsids)
Small icosahedral particles (20-25 nm in diameter)
Natively packaged ssDNA to ~ 4.7 kb
Replication-defective, non-enveloped virus
Non-pathogenic, mildly immunogenic; Low level integration, maintained episomally
Many distinct serotypes

Examples of AAV physical attributes
Capsid purity
Capsid identity
Vector particle titer
Empty/full capsid

Key Structural Characteristics of AAV Products



Case 1: Capsid protein characterization

- Capsid serotype ID
- Sequence coverage
- Sites and types of PTMs

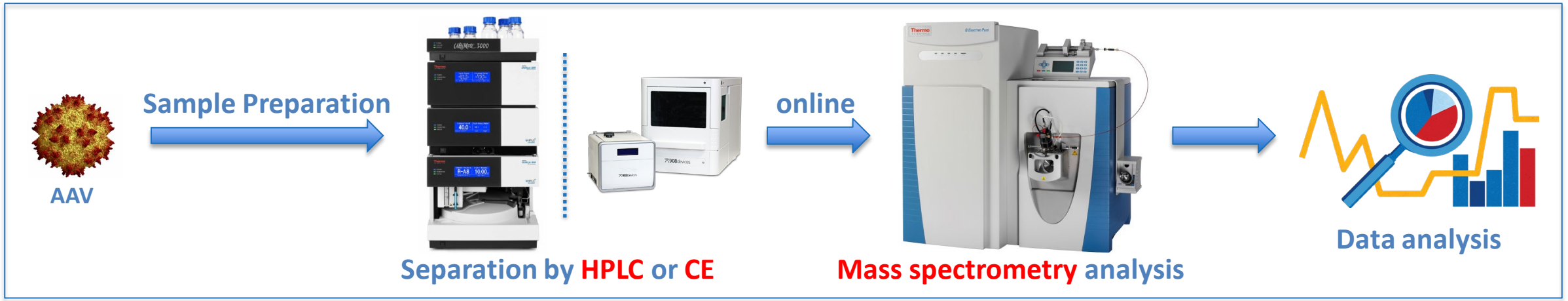
Case 3: Intact AAV analysis

- Empty/partial/full vector
- HMW

Case 2: Encapsidated DNA Characterization

- Genome integrity Assessment

Challenges in Gene Therapy Mass Spectrometry (MS) Analysis



- **AAV is large in size and has complex heterogeneity**
  - ❖ Analyzing intact AAV in native state can provide rich information but requires advanced instruments with higher mass range and/or charge detection capability.
  - ❖ Heterogeneity could be introduced by capsid purity, genome integrity, and/or packaging behavior, etc.
- **Historical knowledge and literatures are limited**
- **Sample availability is limited, and sample concentration is low**

Case Study 1: Characterization of Capsid Proteins by Peptide Mapping

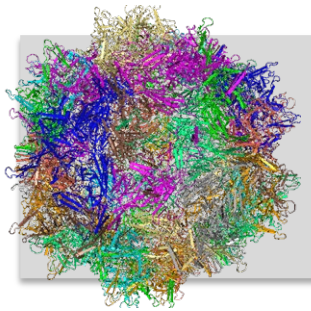
LC-MS Peptide Mapping Workflow

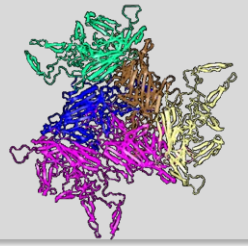
AAVs

- Denaturation
- Reduction
- Alkylation

- Enzymes

- Peptide mixture





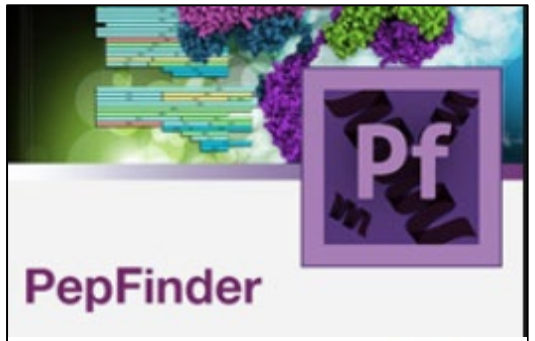
Inject into microLC system to separate peptide mixture

Peptides analyzed by microLC-MS/MS on Q Exactive Plus MS

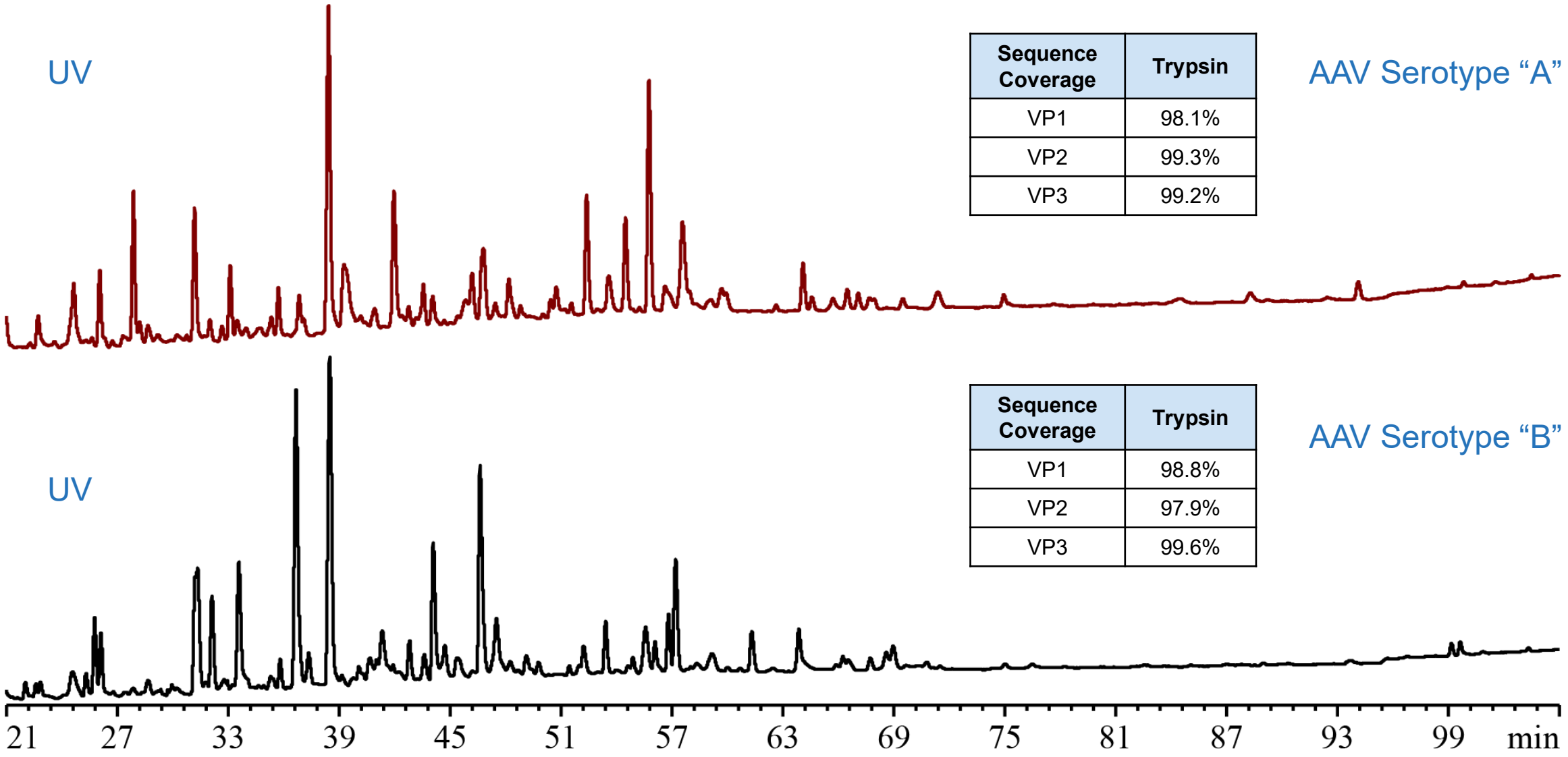
Peptide map

- Sequence coverage
- Sites and types of PTMs
- Unique peptides for ID
- Sequence variants

Data analysis:
PepFinder and Expressionist



Peptide Mapping of Capsid Proteins



Sequence Coverage	Trypsin
VP1	98.1%
VP2	99.3%
VP3	99.2%

AAV Serotype “A”

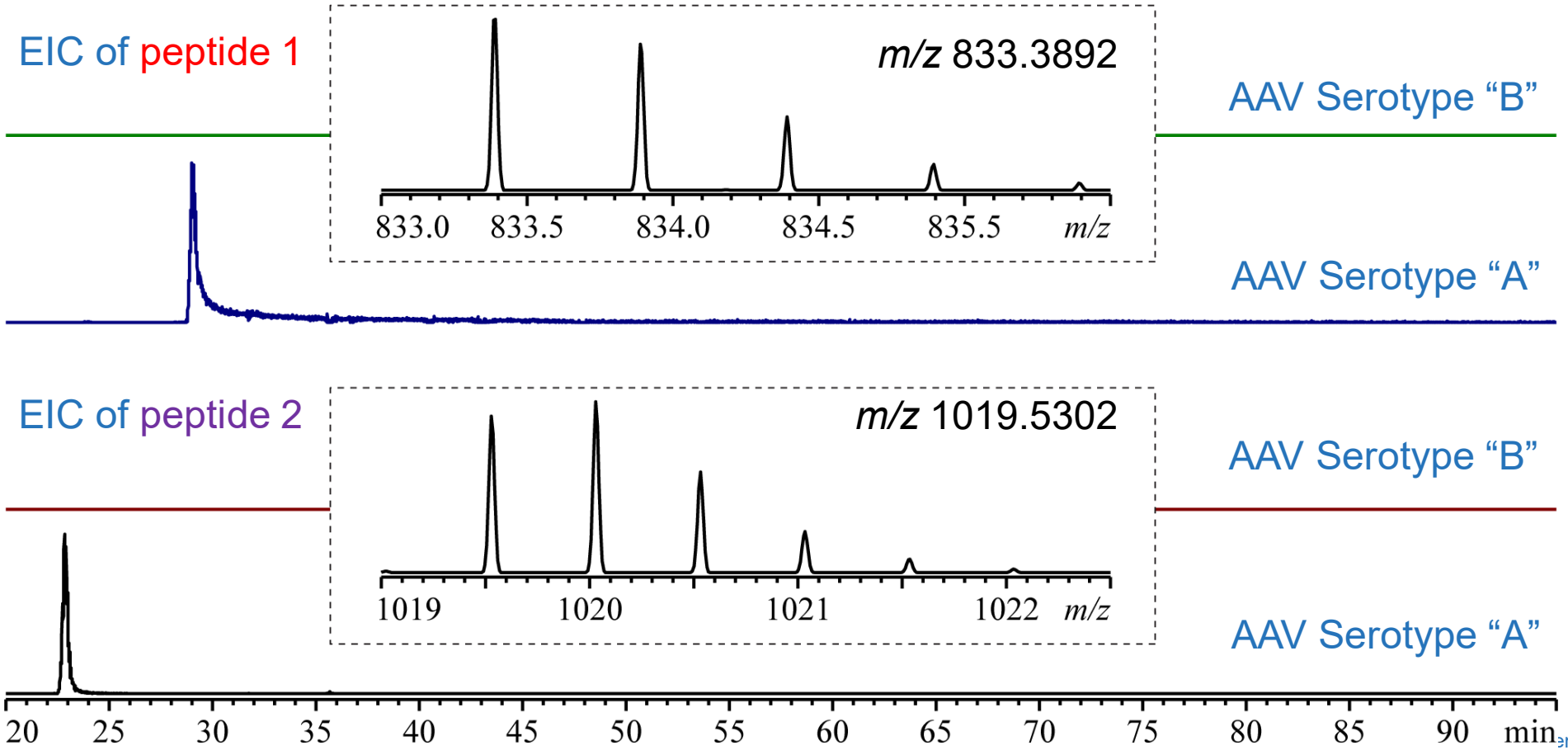
Sequence Coverage	Trypsin
VP1	98.8%
VP2	97.9%
VP3	99.6%

AAV Serotype “B”

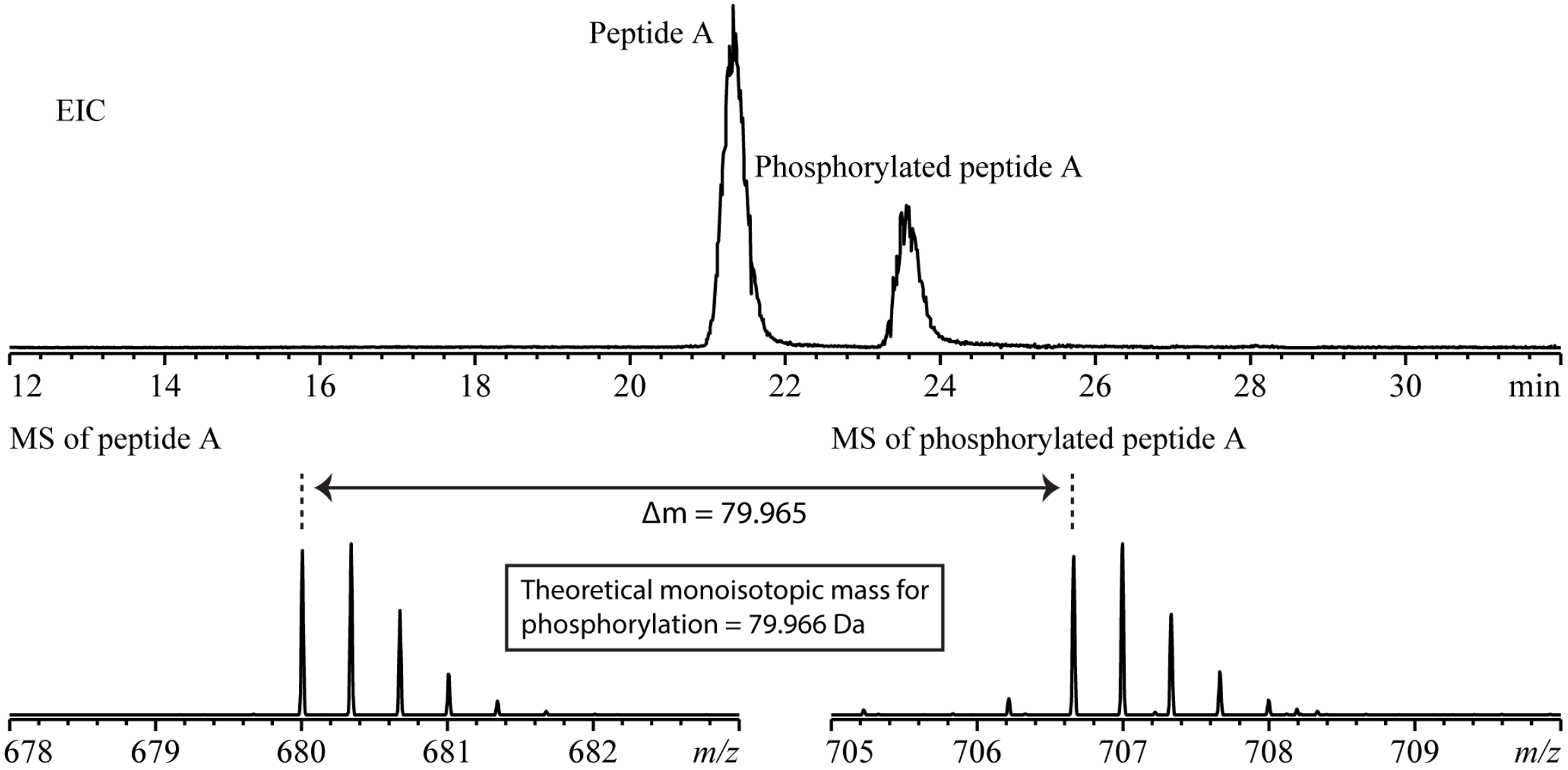
100% sequence coverage was achieved by combination of trypsin, Lys-C and Asp-N peptide mapping.

AAV Serotype Identification by Peptide Mapping

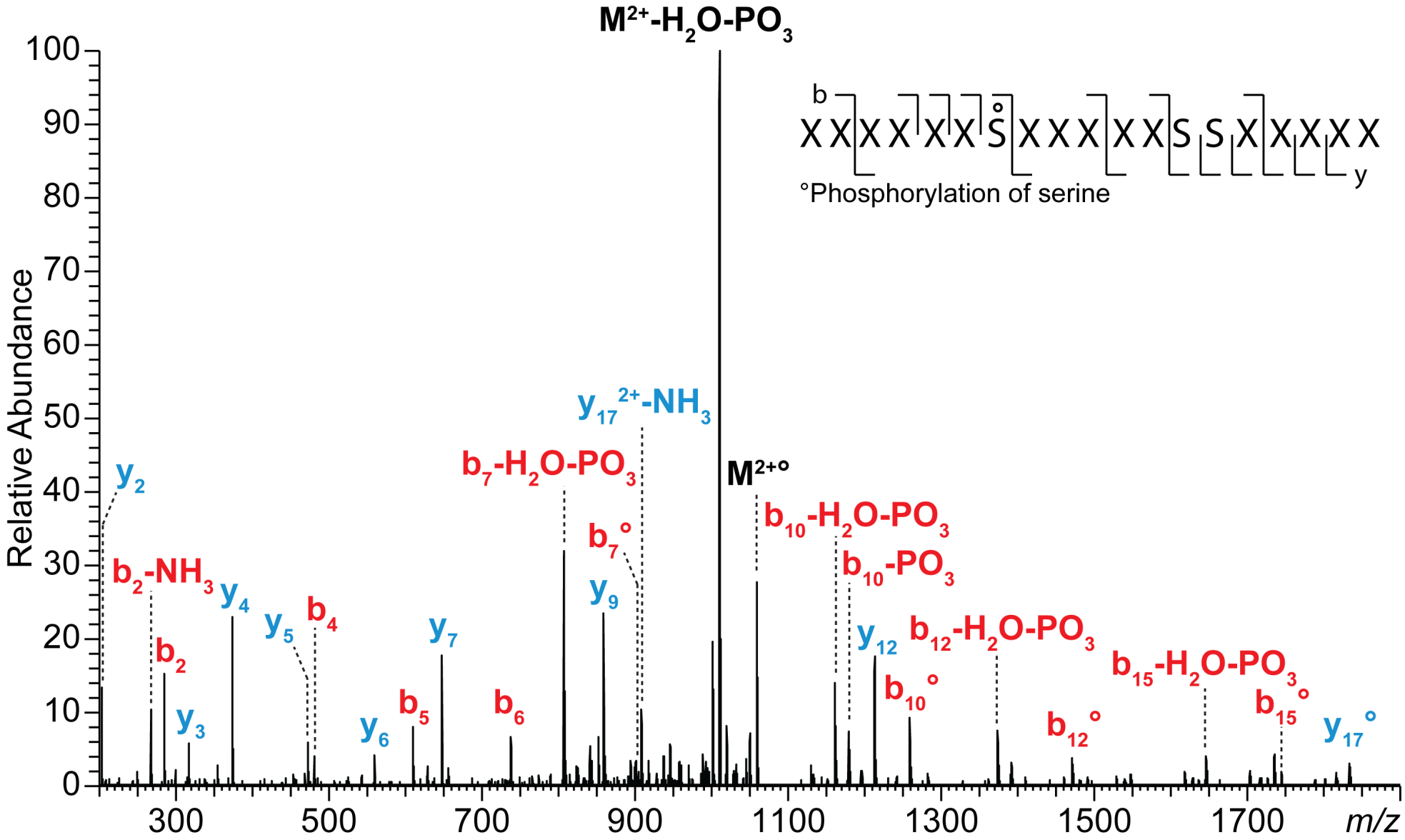
AAV Serotype "A"	Unique Peptide	Sequence	Retention Time (min)	Theoretical Monoisotopic Mass, Da	Charge z	Theoretical *m/z*
	1	GEPXXXXXAAALEHDK	29.1	1664.76	2	833.3894
					3	555.9287
	2	KRPVEQSPQEPDXXXXXGK	22.9	2037.05	2	1019.5319
					3	680.0237



AAV PTM Characterization by Peptide Mapping



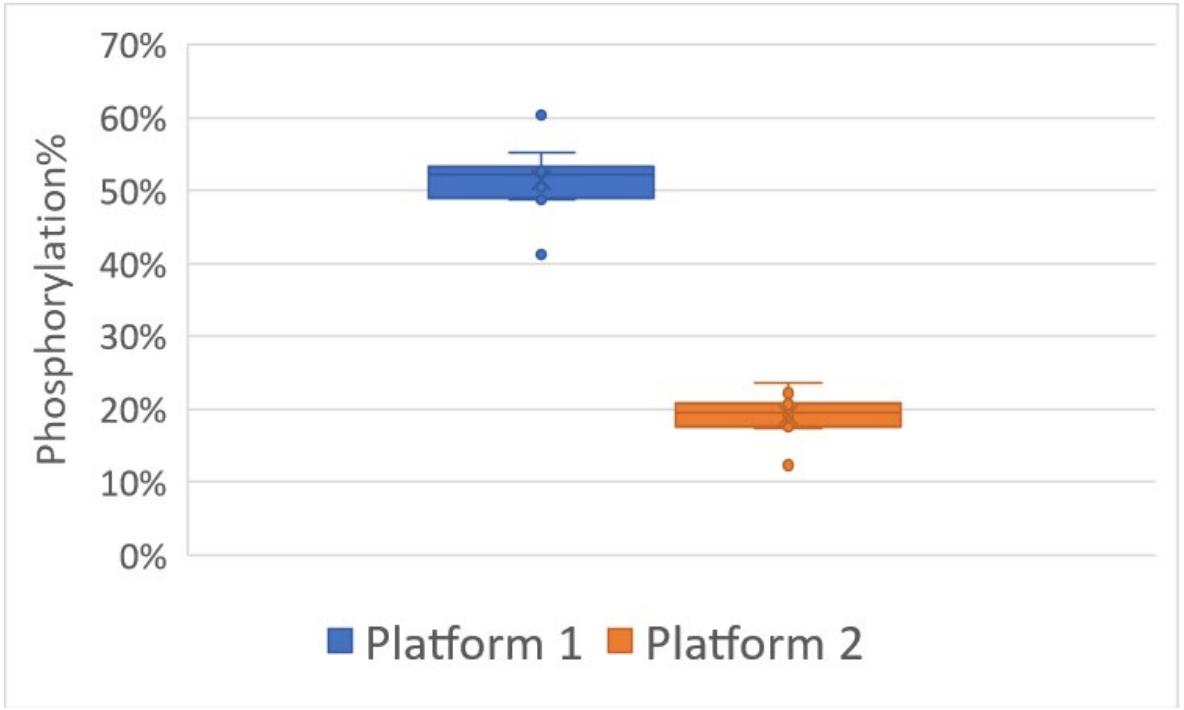
AAV PTM Characterization by Peptide Mapping



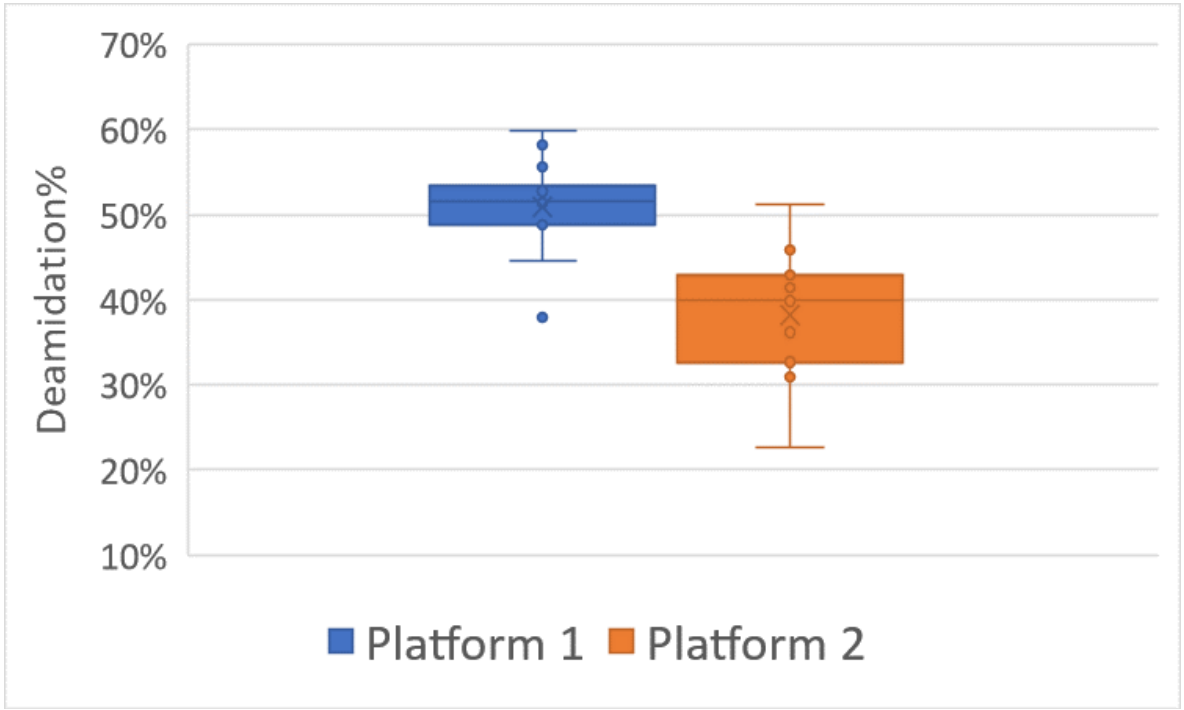
MS/MS confirmed the sites of modifications.

Different levels of PTMs observed for two AAV production platforms

Ser(xxx) Phosphorylation



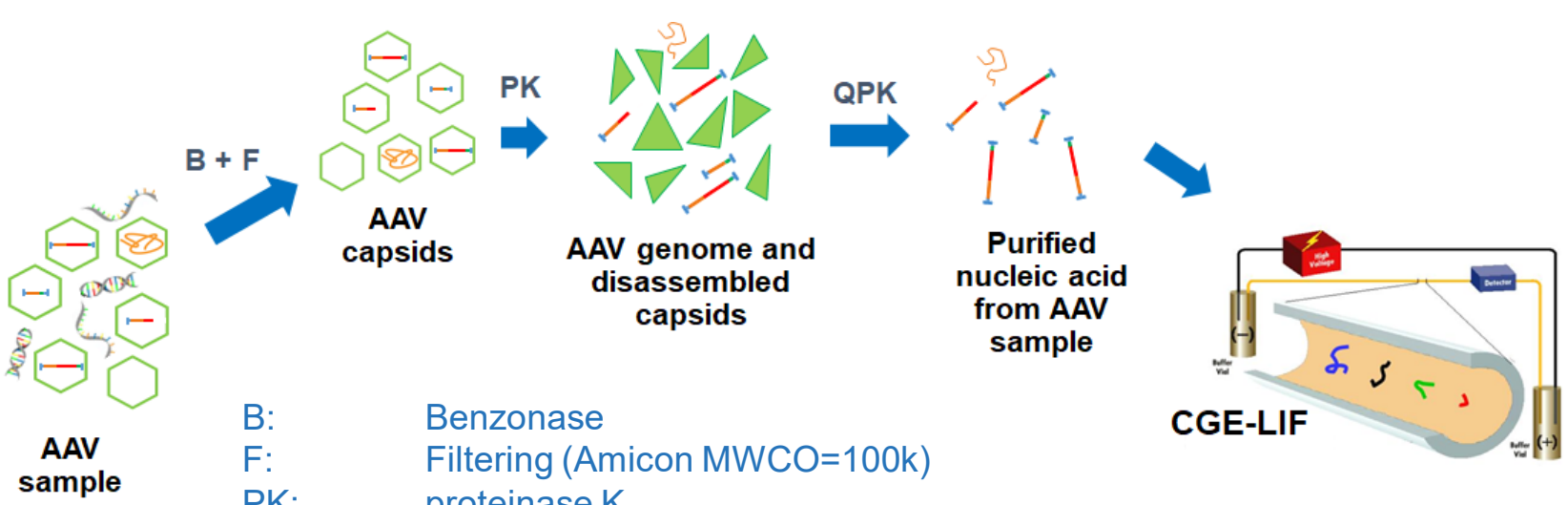
Asn(yyy) Deamidation



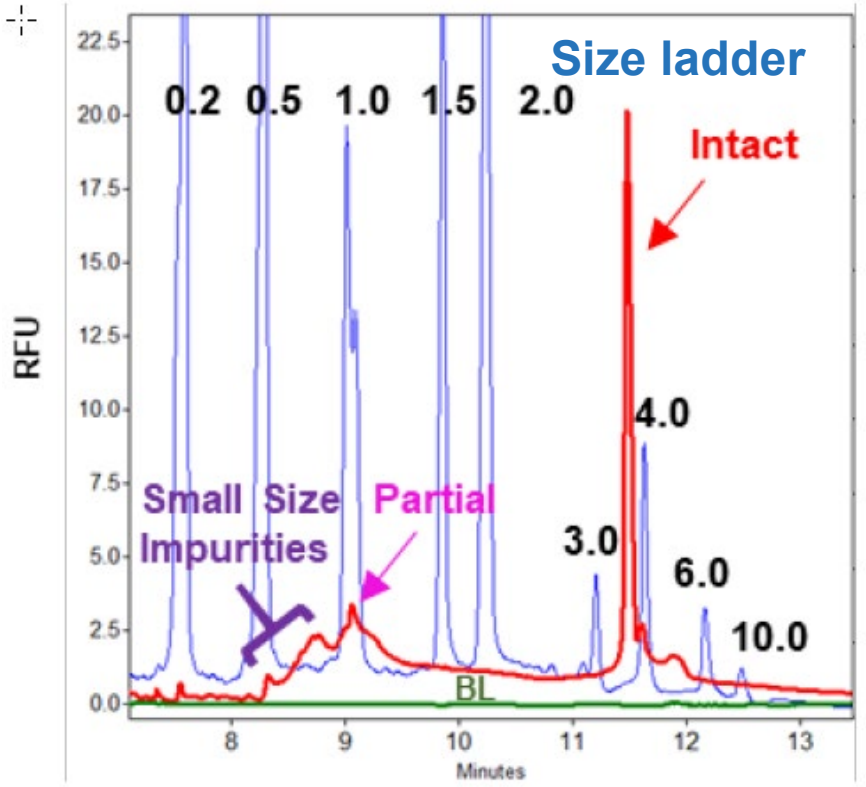
Sample Description	potency (%)	expression (%)
Platform 1 representative	73	66
Platform 2 representative	113	102
Platform 2 (N to D mutant; Asp(yyy))	52	<50

Case Study 2: Genome Integrity Analysis by Capillary Electrophoresis (CE)

Overview of genome integrity workflow

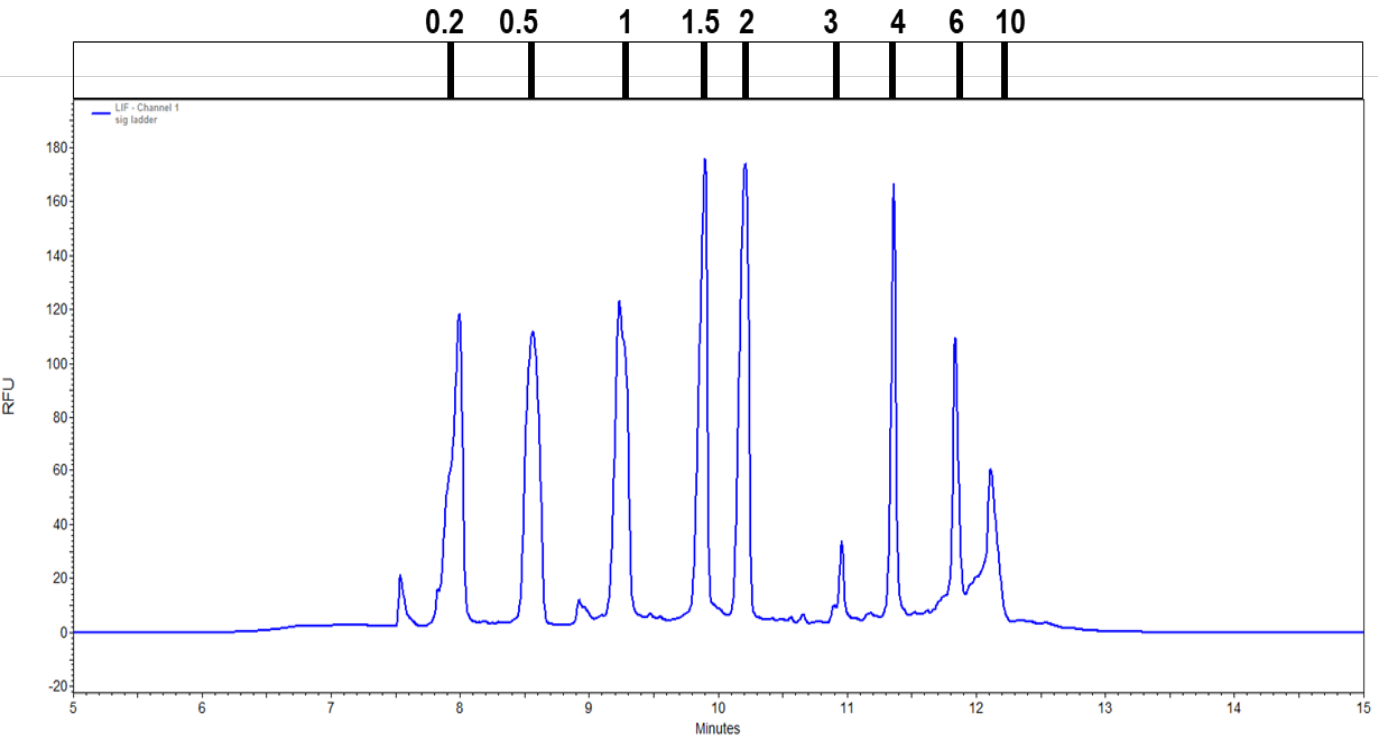


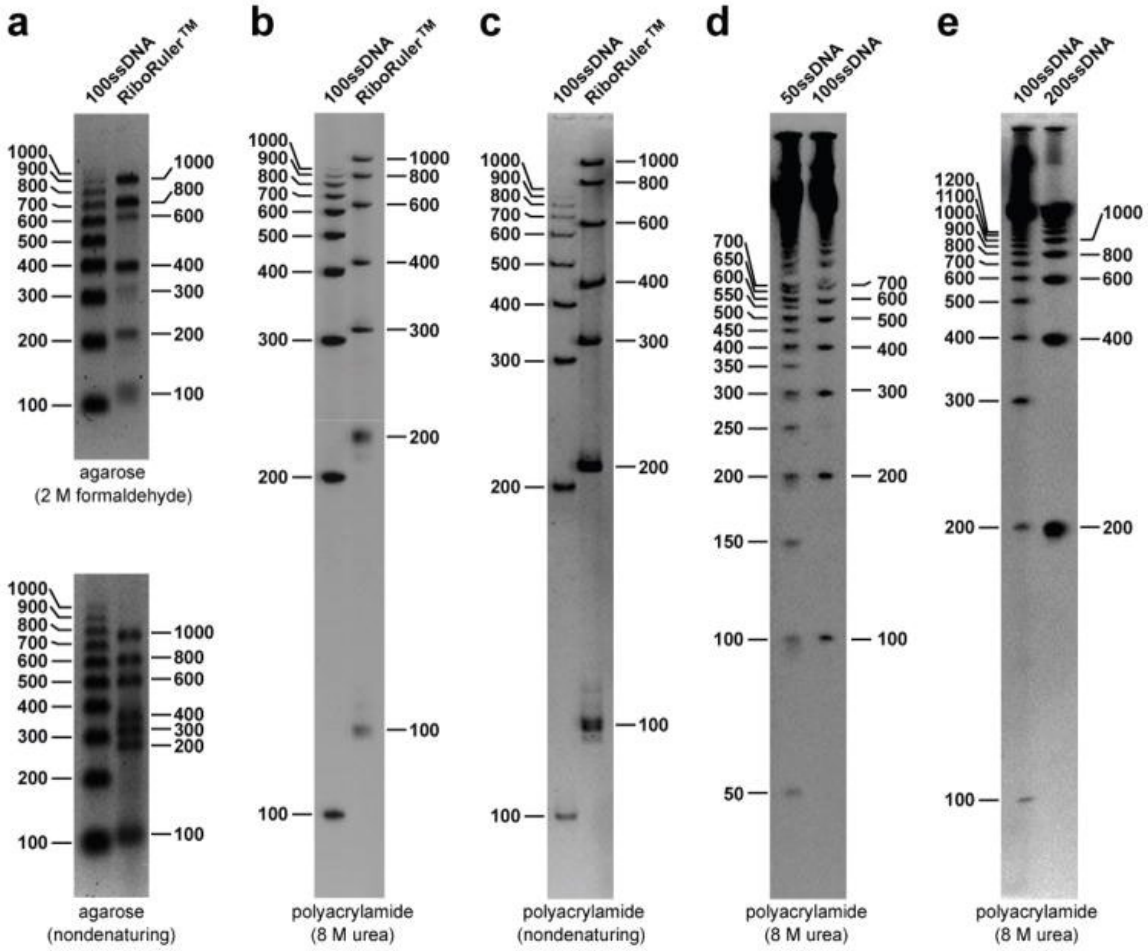
B: Benzonase
F: Filtering (Amicon MWCO=100k)
PK: proteinase K
QPK: QIAquick PCR purification kit
CGE-LIF: Capillary electrophoresis with laser-induced fluorescence detection and SYBR Green II



Figures adapted from: Fang Wang at SCIEX

The ladder for size determination



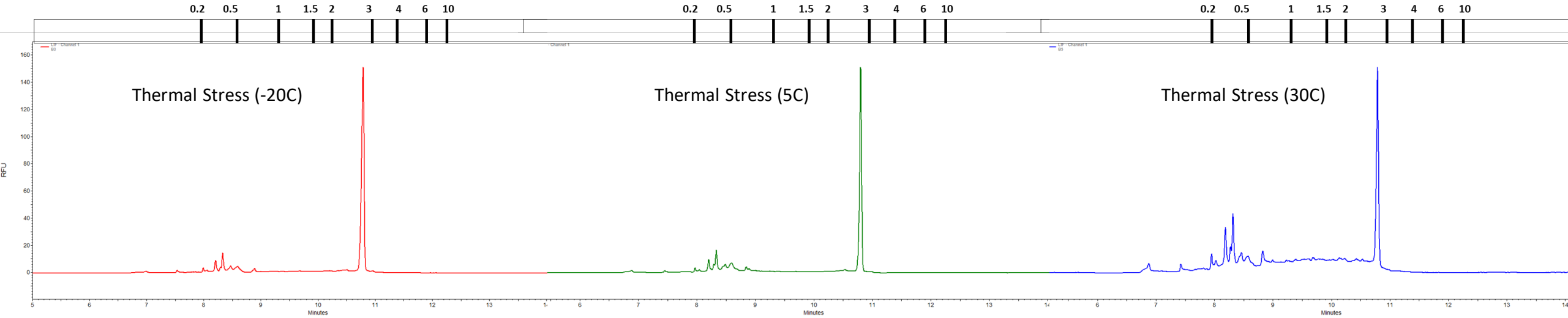


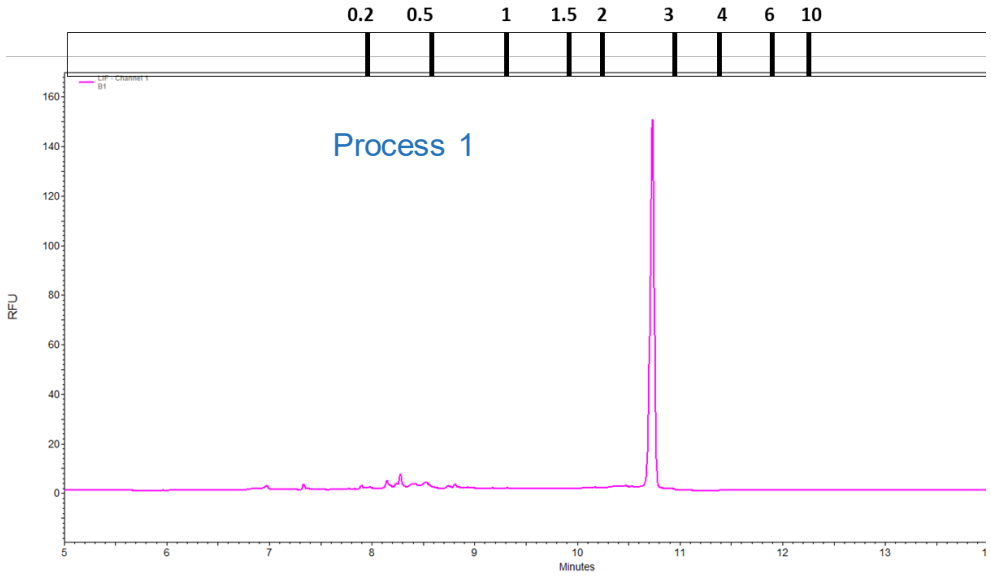
Gu H, Breaker RR. Production of single-stranded DNAs by self-cleavage of rolling-circle amplification products. Biotechniques. 2013 Jun;54(6):337-43

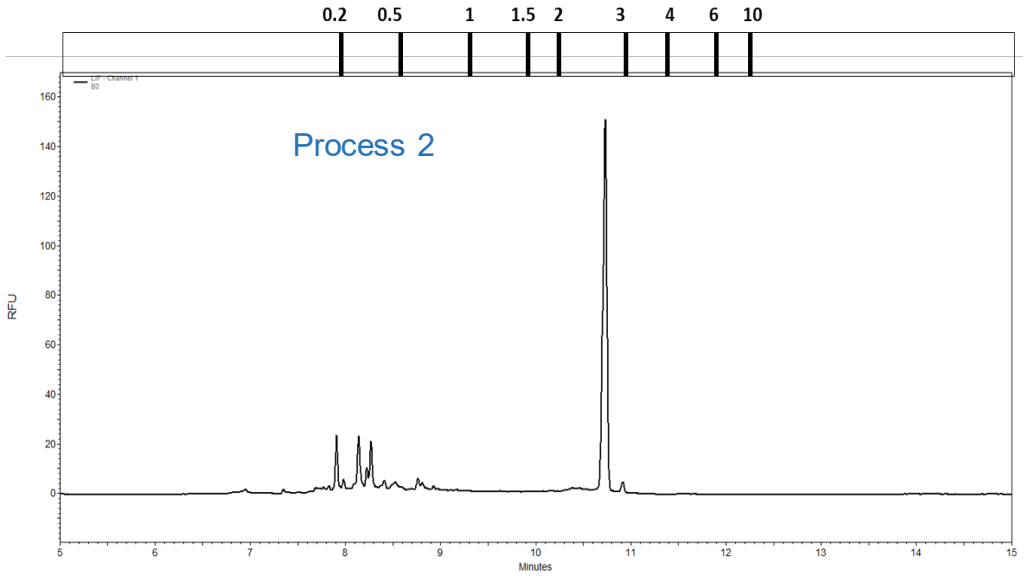
- Left: DNA ladder
- Right: RNA ladder

Figures adapted from: Fang Wang at SCIEX

AAV Genome integrity analysis

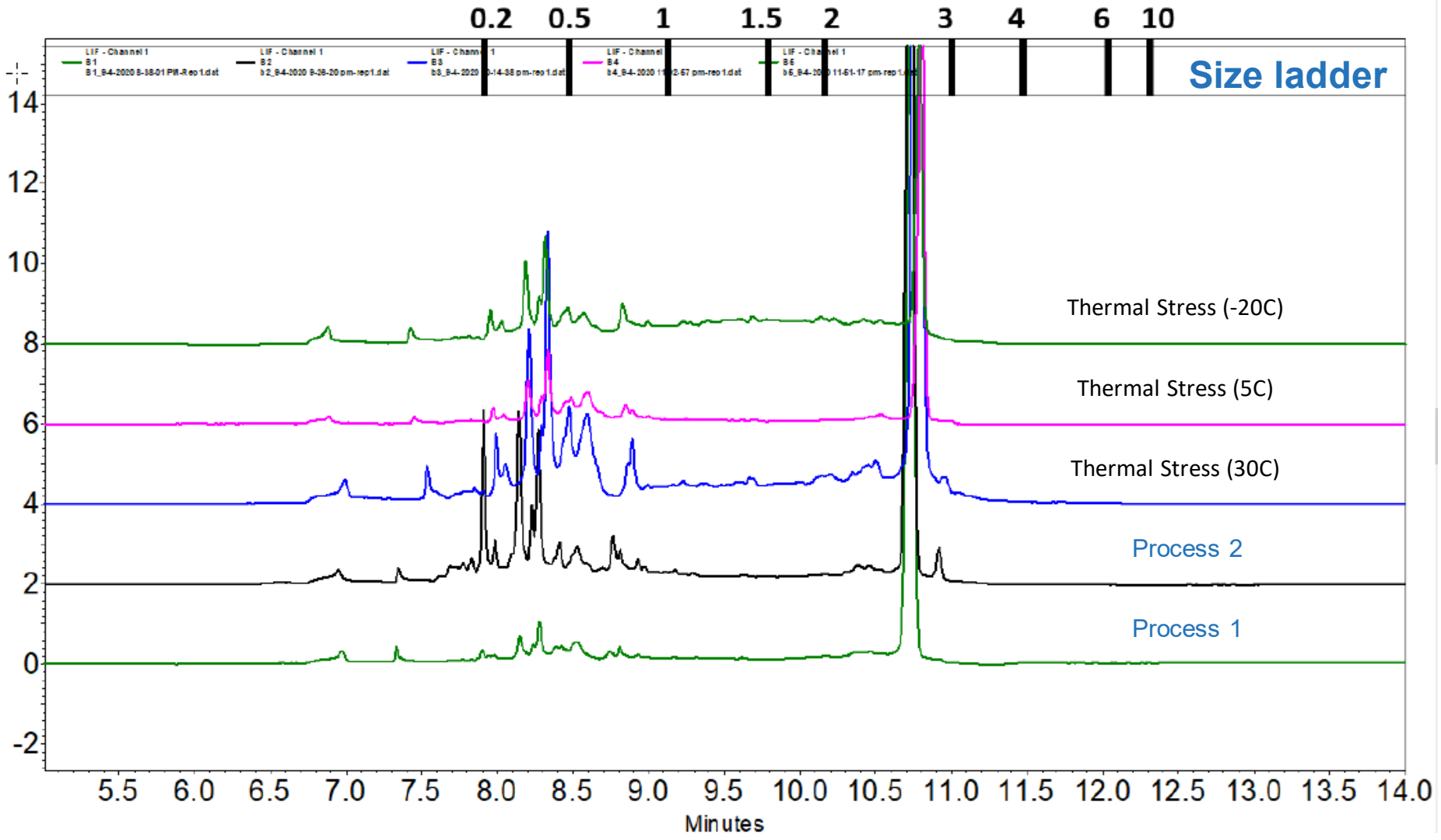






CE data provided by SCIEX

AAV Genome integrity analysis

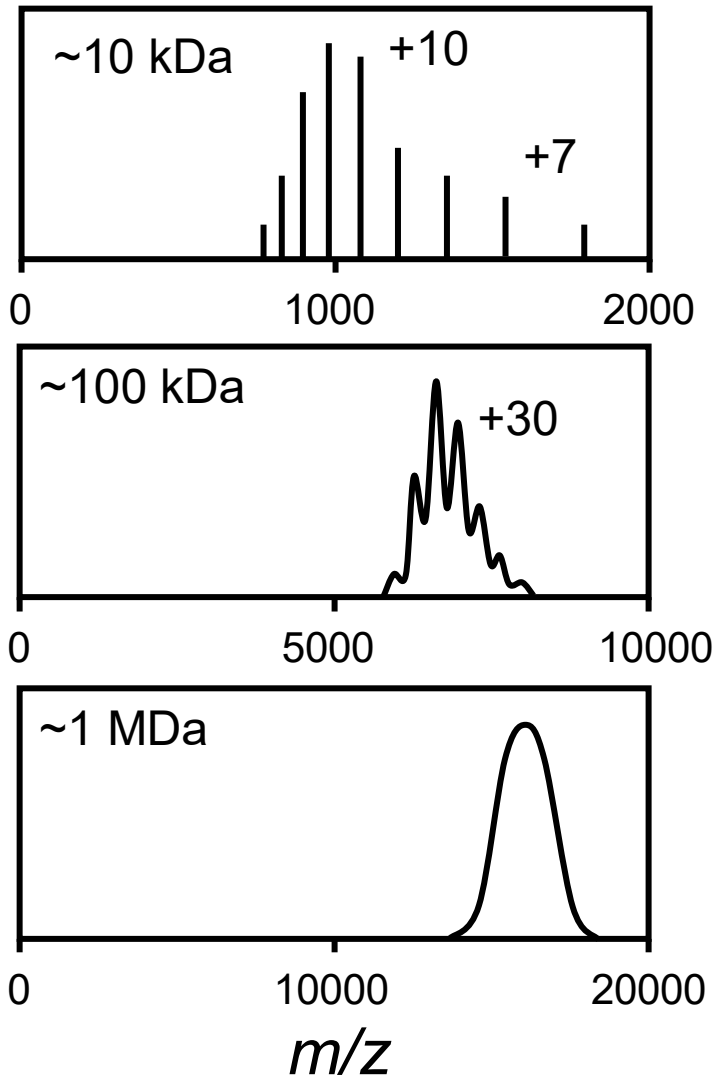


- Thermo stress at 30°C led to significant degradation of genome.
- Different processes had impact on genome integrity
- CE results aligned with NGS data

CE data provided by SCIEX

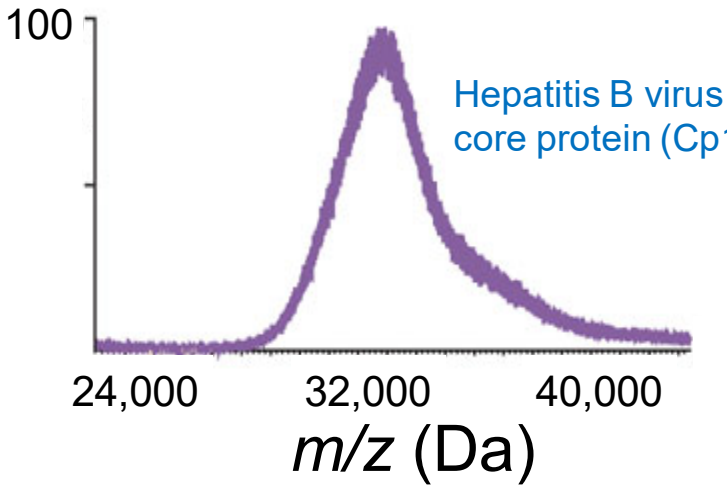
Case Study 3: Characterization of AAV Empty/Full Capsids by Charge Detection MS (CDMS)

Loss of Charge State Resolution of Large Molecules



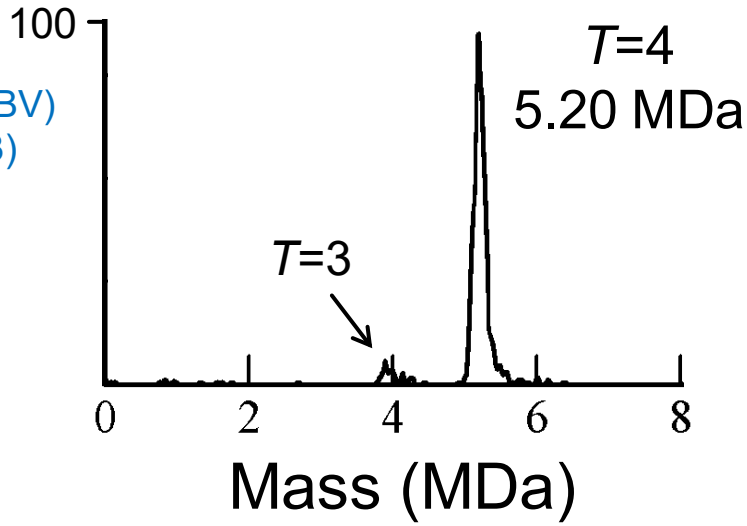
Conventional *m/z* spectrum

- Lack of charge state resolution of large molecules caused by heterogeneity
- *m* could not be determined



Charge detection MS (CDMS)

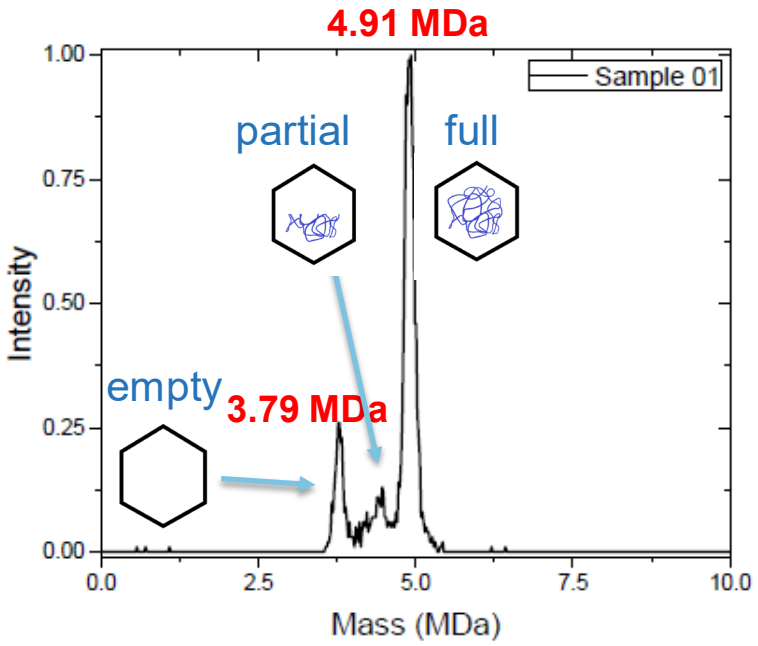
- Measure *m*/*z* and *z* for each ion
- *m*/*z* × *z* → *m* for each ion

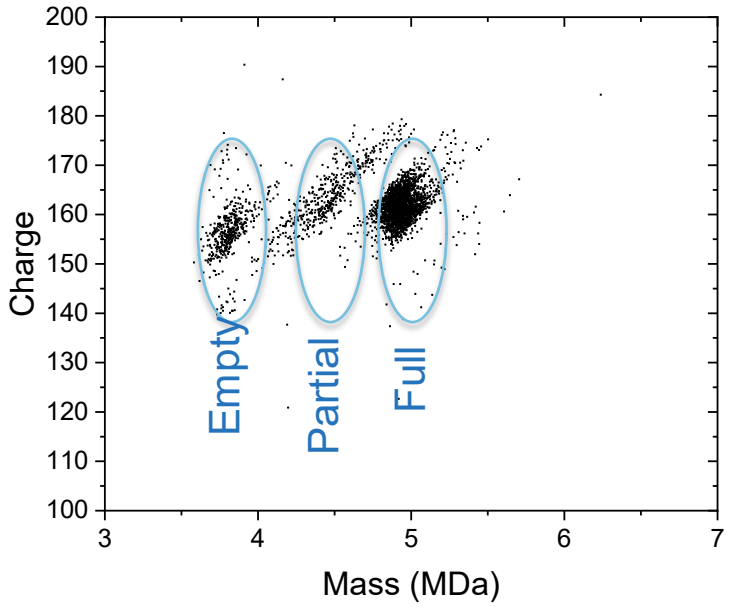


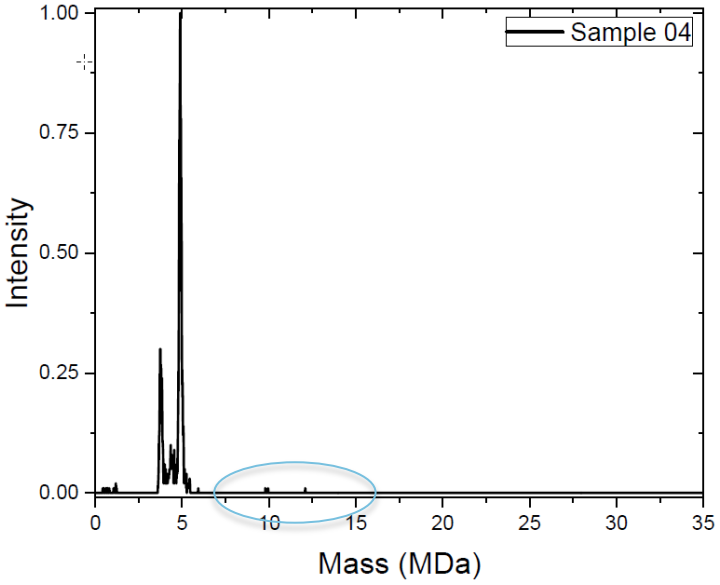
T: triangulation numbers

Figures adapted from: Benjamin E. Draper at Megadalton Solutions

CDMS of AAV







- Two primary populations of capsids detected corresponding to empty and full particles
- Some “intermediate” (partially filled) particles observed

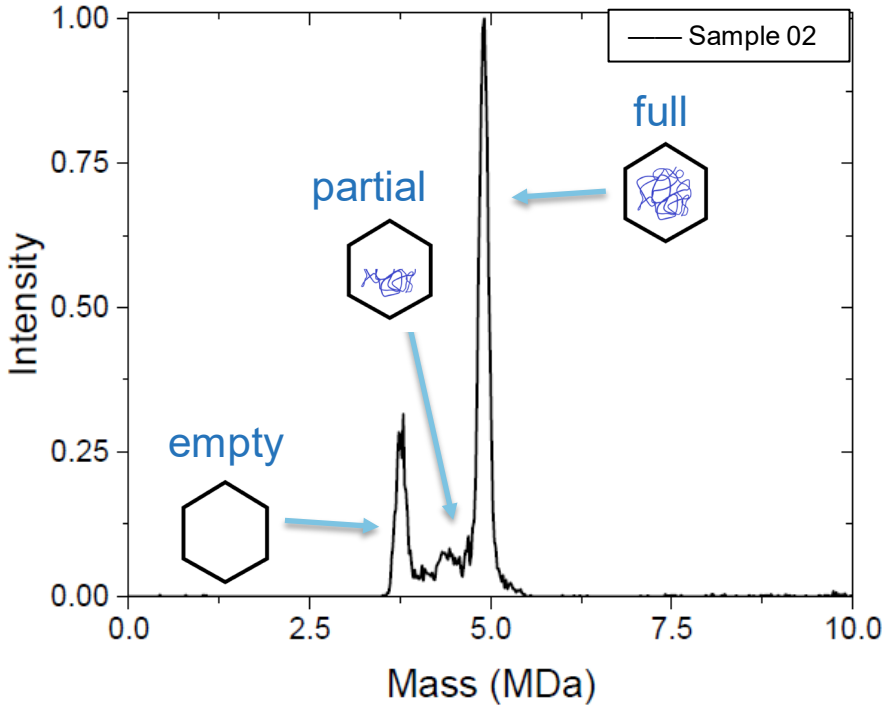
- Empty, partial, and full capsids have similar charge characteristics.

- High-molecular-weight (HMW) species could be characterized.

CDMS data provided by Megadalton Solutions

CDMS and Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

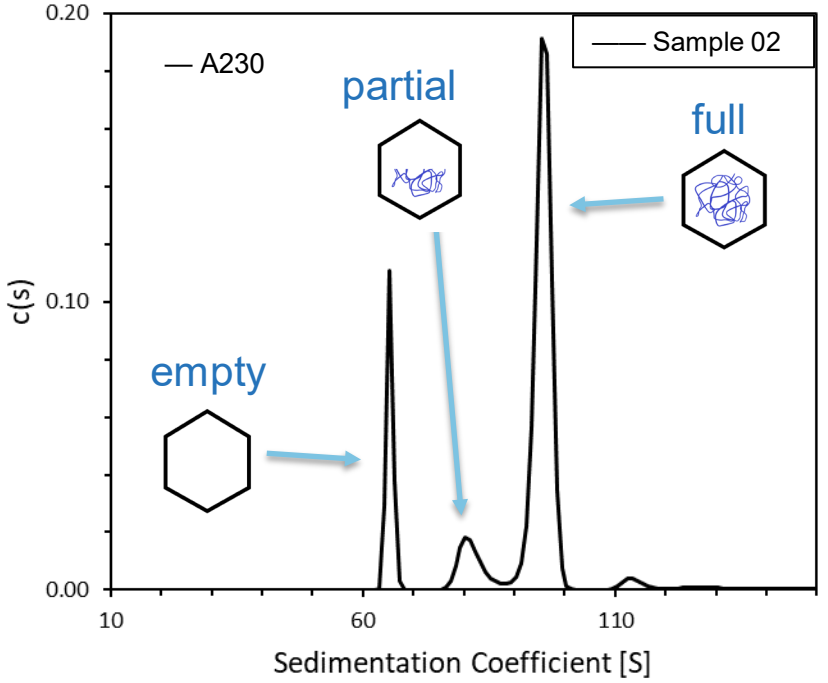
CDMS



Simultaneously measure m/z (mass to charge ratio) and z (charge)

- Resolves intermediate species
- Provide masses of particles
- Provide charge for each species
- Instrument not commercially available yet

SV-AUC



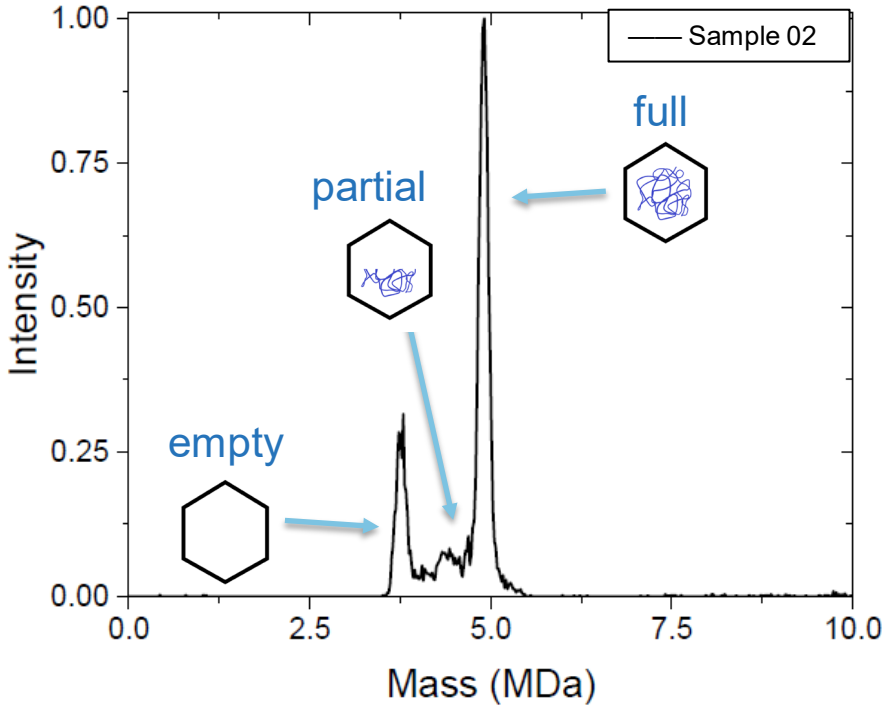
Separate and quantify based on size, shape and mass

- Resolves intermediate species
- Commercial instrument
- High sample amount required
- Low throughput
- Labor intensive

CDMS data provided by Megadalton Solutions

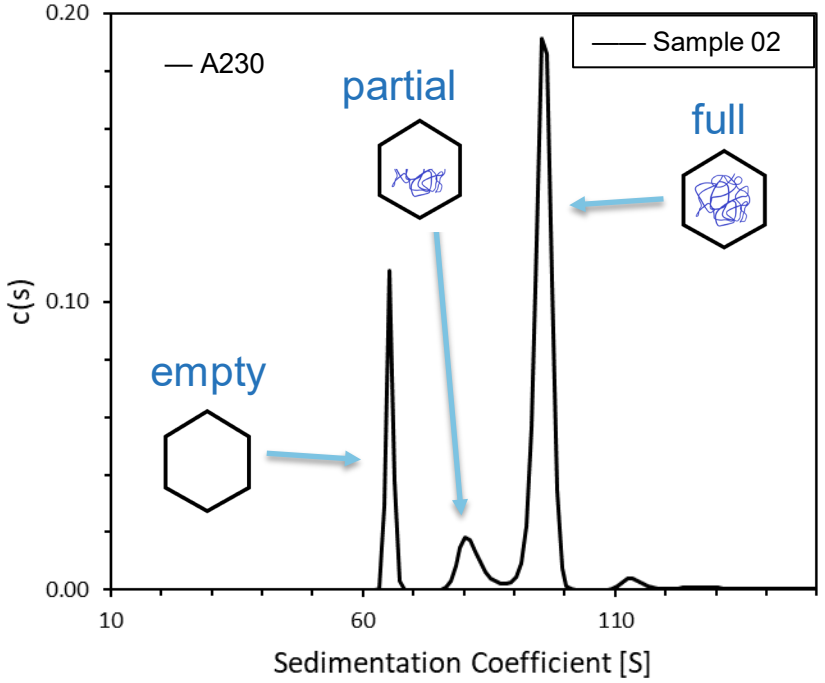
CDMS and Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

CDMS



LMW	0.1%
Empty	19.4%
Partial	14.2%
Full	65.2%
HMW	1.1%

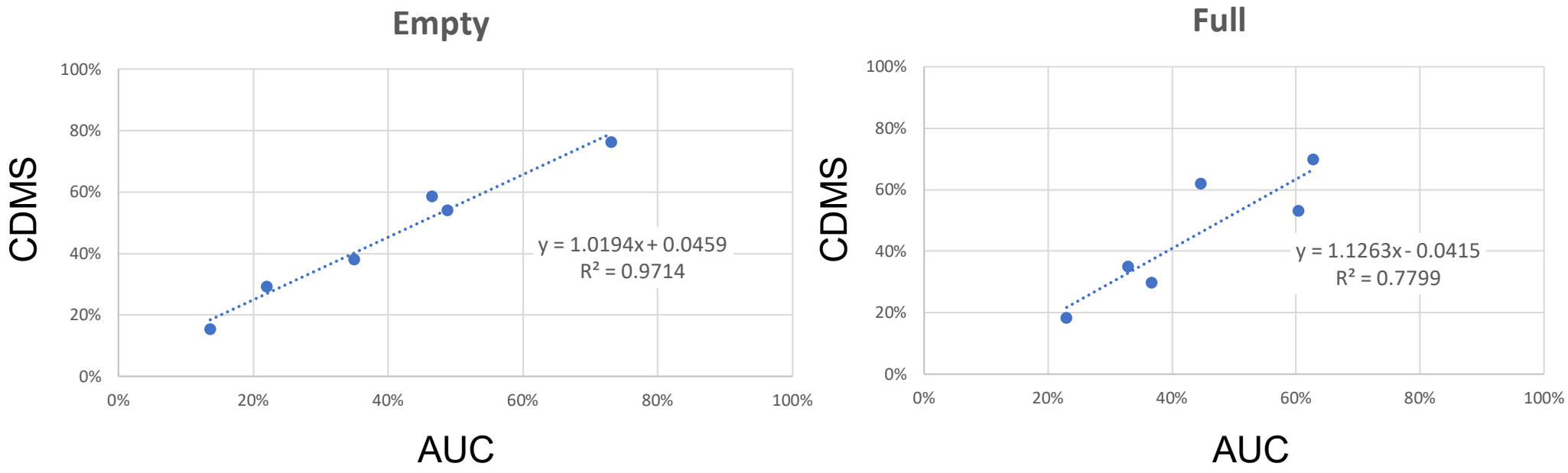
SV-AUC



LMW	0%
Empty	16.7%
Partial	9.6%
Full	68.9%
HMW	4.8%

CDMS data provided by Megadalton Solutions

Good Correlation between AUC and CDMS for Empty and Full



CDMS is a suitable method for quantifying %empty and %full capsids

Orthogonal Empty/Full Methods

Method	AUC	CDMS	TEM	IEX
Sample Concentration (VG/mL)	~E+12	~E+9	~E+12	~E+12
Volume requirements	high	Intermediate	low	Intermediate
Throughput	low	Intermediate	low	high
Sample preparation	Extensive	Intermediate	Easy	Easy
Personnel training	Extensive	Extensive	Intermediate	Easy
QC Readiness	No	Potentially Yes	No	Yes
Attributes	All	All	Empty, Full	Empty, Full

Method (E/F)	AUC			CDMS			TEM		IEX	
	%Empty	%Full	%Partial	%Empty	%Full	%Partial	%Empty	%Full	%Empty	%Full
Sample 03	42.3	31.9	17.0	52.5	27.9	19.7	55	45	58.5	41.5

Conclusions

- ***CDMS*** is a powerful novel analytical tool that shows great promise in AAV-based gene therapy development.
- ***CE*** coupled with fluorescence detection provides the capability to assess AAV genome integrity quickly.
- Varying levels of PTMs were observed by ***peptide mapping*** for different AAV production platforms.
- Unique peptide detection could be used for AAV serotype identification.
- Deamidation could impact potency of AAV gene therapy products; higher deamidation correlated to lower potency.

Acknowledgements

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