APPLICATION NOTE 000253

Highly sensitive intact mass analysis of AAV capsid proteins using a UHPLC-FLD-HRAM MS platform

Authors: Reiko Kiyonami[1], Kristina Srzentić[2], Rosa Viner[1], Weijing Liu[1], Min Du[2], Katy Irvin[3], Kenneth Thompson[3], Chao Yan Liu[3], Jon Zmuda[3]

[1] Thermo Fisher Scientific, San Jose, CA;
[2] Thermo Fisher Scientific, Cambridge, MA
[3] Thermo Fisher Scientific, Frederick, MD

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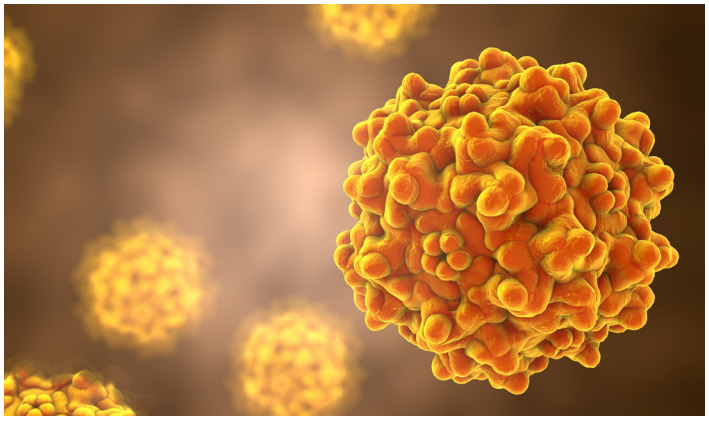
Application benefits

- Highly confident adeno-associated virus (AAV) capsid protein characterization using a UHPLC-FLD-HRAM MS platform.
- Excellent sensitivity and mass accuracy for intact mass confirmation of viral proteins and their proteoforms using HRAM intact mass analysis.



- Confident sequence confirmation of low abundant viral protein proteoforms by high performance top-down analysis.
- Rapid and sensitive assessment of the ratio of the AAV structural proteins VP1:VP2:VP3 using fluorescence detection.

Goals

- Demonstrate the benefits of excellent spectral quality as well as high sensitivity enabled by the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer for accurate AAV capsid protein intact mass analysis.
- Demonstrate the benefits of the top-down approach for sequence confirmation of all proteoforms and confirmation/elucidation of low abundant ones as well.

Introduction

Recombinant adeno-associated viral (rAAV) vectors have emerged as the leading gene delivery vehicles for gene therapy due to their high-efficiency transduction and safety.[1,2] A wild-type AAV particle contains a single-stranded DNA genome and a capsid comprised of three structural viral proteins (VP1, VP2, and VP3) that assemble the viral particle at an approximate ratio of 1:1:10.[1] Each VP has an overlapping sequence at the C-terminus and differs only at its N-terminal region.[4,5] The surface composition of the AAV capsid is an essential component that is involved in cell binding, internalization, and trafficking within the targeted cell.[5] Complete characterization of viral proteins is required to ensure the safety, quality, and efficacy of AAV products.[6] High performance liquid chromatograph-mass spectrometry (HPLC-MS) technology enables faster, more sensitive, and more quantitative approaches for capsid protein characterization and is emerging as a fast alternative analytical method for AAV capsid protein characterization.[7,8] Unlike the therapeutic proteins, the AAV sample is more complex in structure and limited in sample volume with low titer due to small batch sizes, yielding a very low amount of capsid proteins for subsequent HPLC-MS analysis. The desired HPLC-MS platform for the characterization of the AAV viral proteins needs to offer high sensitivity for detecting low abundant viral proteins and provide accurate intact mass measurement of these viral proteins.

Here we demonstrate that an UHPLC-Orbitrap MS platform can be used to characterize the AAV capsid proteins and their associated variants with high sensitivity and unparalleled mass accuracy. As a case study, we analyzed AAV Serotype 6 sample expressed in HEK293 cell line on an HPLC-Orbitrap MS platform. The high sensitivity and ultra-high resolution offered by the Orbitrap mass spectrometer enabled excellent spectral quality for denatured AAV protein intact mass analysis. The high sensitivity and ultra-high resolution and accurate mass of the Orbitrap MS also enabled confident sequence identification of truncated VP3 forms through top-down analysis.

Experimental

Sample preparation

AAV6 sample was expressed via transient transfection in HEK293 cells. The titer of the AAV6 sample after purification was determined using qPCR to be 2.9E13 vg/mL. The AAV sample was buffer exchanged and concentrated into 80% H_2O/20% acetonitrile containing 5 mM TCEP and 0.1% formic acid using 30K centrifugal filter (Amicon® Ultra, 0.5 mL). The collected sample was incubated at room temperature for 2 hours. The estimated protein concentration for the concentrated AAV sample was 0.28 µg/µL.

Chromatography

For all experiments chromatographic separations were carried out using a C4 stationary phase column (1.0 x 100 mm, 300 Å, 1.7 µm) on the Thermo Scientific™ Vanquish™ Horizon UHPLC system, consisting of the following modules:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A)
- Thermo Scientific™ Vanquish™ Fluorescence Detector F equipped with a Vanquish Fluorescence Micro Flow Cell (P/N VF-D50-A)

Solvent A: Water with 0.1% difluoroacetic acid
Solvent B: Acetonitrile with 0.1% difluoroacetic acid

Chromatographic separation details are summarized in Table 1.

The column temperature was set to 80 °C. The fluorescence detector (FLD) settings are shown in Table 2. For intact mass analysis, sample volumes injected were 2 µL, 0.5 µL, 0.2 µL and 0.1 µL. For top-down analysis, the sample volume injected was 10 µL.

Table 1. Overview of LC flow rate and applied gradient used for MS analysis of intact viral proteins under denaturing conditions

Time	Flow (mL/min)	%B
0	0.05	20
1	0.05	32
16	0.05	36
20	0.05	80
21.5	0.05	80
22	0.05	20
30	0.05	20

Table 2. FLD settings used for analysis of intact viral proteins under denaturing conditions

Excitation wavelength	280 nm
Emission wavelength	350 nm
Lamp mode	High power
Sensitivity	1
Data collection rate	5 Hz

Mass spectrometry

The Orbitrap Exploris 480 mass spectrometer (P/N BRE725533) equipped with the BioPharma Option (P/N BRE725539) was used for MS data collection. The instrument was operated with Thermo Scientific™ Xcalibur™ 4.2 SP1 software and controlled by Orbitrap Exploris Series 3.0 instrument control software. MS settings for all experiments presented here are shown in Table 3.

Table 3. MS parameter settings

MS conditions	Intact	Top-down
Method type	Full MS	tMS^2
Scan range (*m/z*)	700-6,000	200-3,000
Application mode	Intact	Intact
Pressure mode	Low	Low
Resolution	15,000 at *m/z* 200	120,000 at *m/z* 200
RF lens (%)	150	150
AGC target value	75	300
Max inject time (MS)	100	200
Isolation window (MS^2)	-	680 Da; 420 Da
Microscans	10	5
Source fragmentation (V)	25	25
HCD collision energy (V)	-	25
	MS source settings	
Sheath gas (a.u.)	25	25
Aux gas (a.u.)	8	8
Sweep gas (a.u.)	0	0
Spray voltage (V)	3,400	3,400
Capillary temp. (°C)	320	320
Vaporizer temp. (°C)	100	100

Data processing

Raw data files were processed with Thermo Scientific™ BioPharma Finder™ 4.1 software. For isotopically unresolved intact protein spectra deconvolution, Sliding Window and ReSpect algorithms included in the intact mass analysis workflow were used. For processing of the top-down spectra and assignments of fragment ions, top-down default deconvolution method provided in BioPharma Finder software was used.

Results and discussion

Intact capsid protein analysis

The denatured AAV6 sample was directly introduced and separated using the C4 column. Figure 1 shows a representative FLD elution profile of detected viral proteins and associated VP3 clip as well as another truncated VP3 form. Using integrated peak areas of three viral proteins (VP1, VP2, and VP3), their respective ratio was estimated as 1.3:1:10.

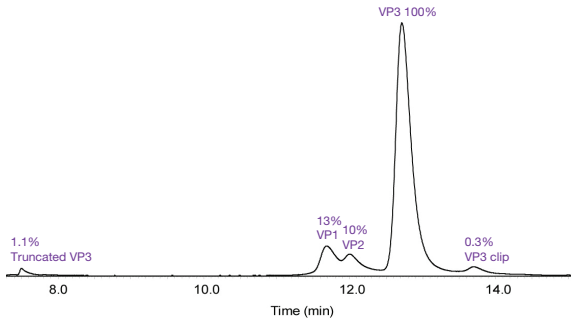


Figure 1. FLD elution profile of AAV6 denatured capsid protein mixture. The integrated FLD peak areas were used for relative quantitation. The relative abundance of the detected components are calculated against the integrated peak area of VP3, which is assigned as 100%.

One of the advantages of using MS-based intact mass measurement is to get direct molecular weight information of viral proteins. The high resolution, high sensitivity, and high desolvation efficiency offered by the Orbitrap Exploris 480 mass spectrometer allowed for unparalleled spectral clarity and, hence, precise average mass measurement. Figure 2 shows the MS raw spectra and deconvoluted results for VP1, VP2, and VP3 obtained from 560 ng viral proteins loaded on chromatographic column (2 µL injection). Exceptional spectral quality was observed not only for VP3 but also for the less abundant VP1 and VP2, all measured with excellent mass accuracy <3 ppm. Figure 3 shows that exceptional spectral quality was also sustained for the very low abundant VP3 clip and truncated VP3 form, and the resulting mass accuracy obtained upon deconvolution was below 10 ppm for low abundant species.

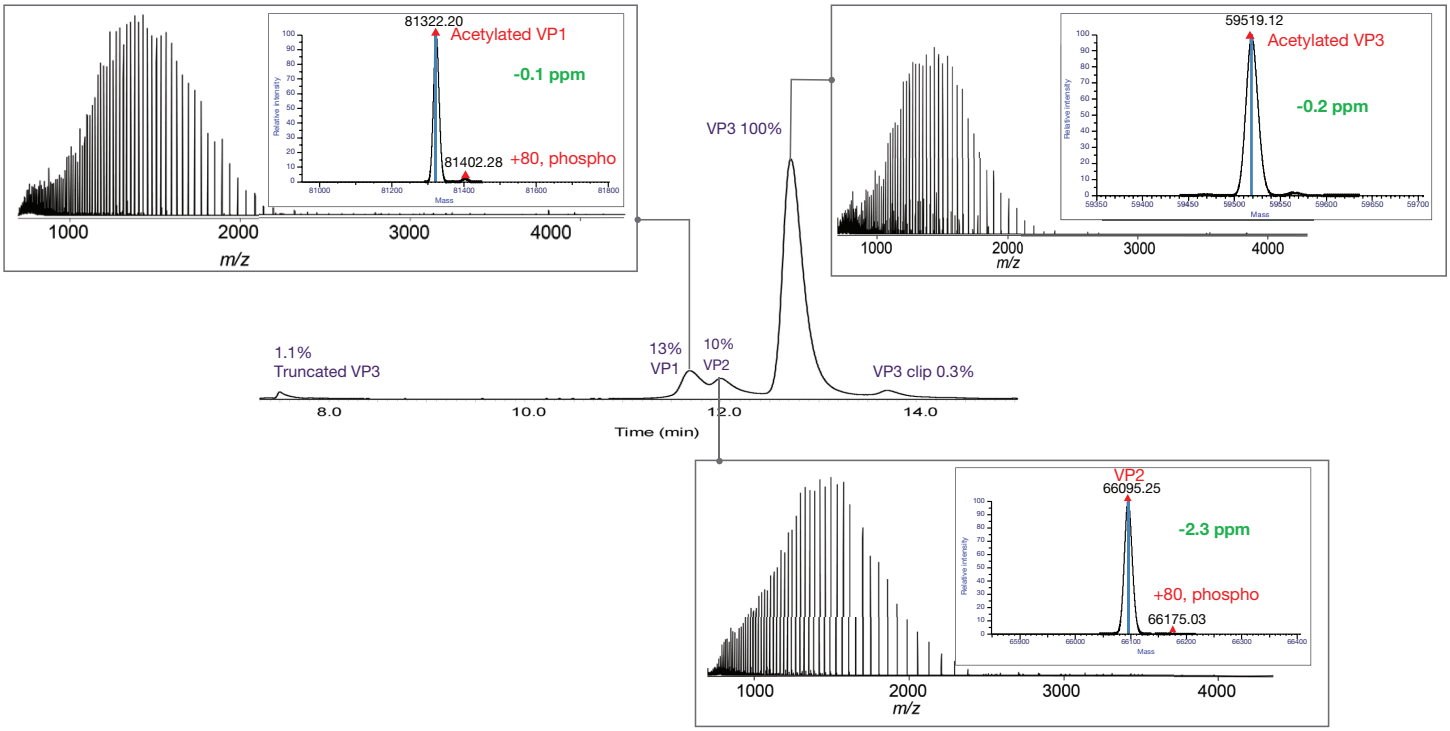


Figure 2. Full MS spectra representing an average of several scans acquired at 10 microscans each from three intact VP proteins (560 ng loaded on column) under denaturing conditions, at a resolution setting of 15,000 (at *m/z* 200). The inset represents the deconvolution result providing highly accurate average mass (below 3 ppm) for all VP proteoforms.

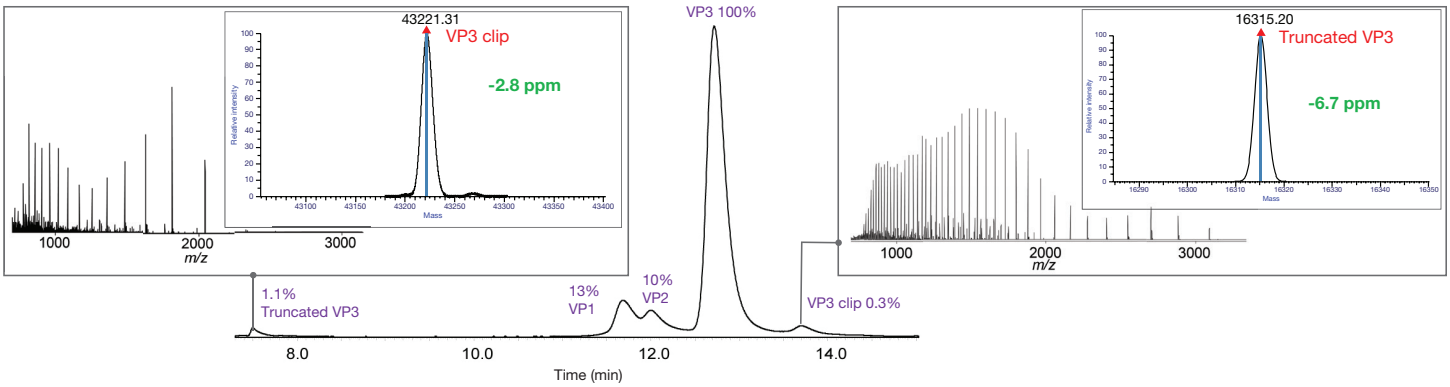


Figure 3. Full MS spectra representing an average of several scans acquired at 10 microscans each from low abundant proteoforms of VP proteins (560 ng on column) under denaturing conditions, at a resolution setting of 15,000 (at *m/z* 200). The inset represents the spectrum obtained upon deconvolution.

Since the AAV sample is generally produced in limited quantities, less sample load requirements for individual analysis is desirable. The excellent performance of the UHPLC-Orbitrap Exploris 480 MS platform allows accurate intact mass analysis even for very low abundant AAV viral proteins. Figure 4 shows the acquired raw spectra and deconvoluted results for VP1 from an injection sequence with increased AAV6 sample injection volumes (0.1 µL, 0.2 µL, 0.5 µL). The estimated amounts of total viral proteins on column from this injection sequence were 28 ng, 56 ng, and 140 ng, respectively. Great spectral quality of MS data was observed across the injection range, and the mass accuracy was less than 3 ppm even at the lowest 28 ng concentration level.

Identification of truncated VP3 proteoforms using top-down approach

It is important to characterize not only major VP proteoforms, but also all associated processed proteoforms to ensure safety and the quality of AAV products. In our AAV6 case study, multiple low abundant proteoforms were detected as shown in Figure 3. One of the proteoforms eluted very close to the VP3 protein (RT 17 min, Figure 3) has an average molecular weight of 43,221.31 Da. Another proteoform that eluted much earlier (RT 9.5 min, Figure 3) has an average mass of 16,315.20 Da. If we sum both these precursors, we obtain an average mass of intact VP3 (43,221 + 16,315.5 = 59,536 Da). The top-down approach was used to confirm the protein sequences of these two proteoforms. The number of experimental strategies for top-down experiments has increased in the past few years due to hardware developments and an increasing number of options made available by the instrument's method editors.[9] The method providing optimal results depends on several key factors: the available instrument and fragmentation modes, the resolution settings at the MS^1 and MS^2 level, the isolation width, the scan speed of the instrument, the amount and complexity of the sample, the infusion or separation conditions, the size and amino acid sequence of the proteins, and whether or not disulfide bridges are present. For the current study, we performed a top-down experiment using wide isolation windows and single fragmentation (HCD) and collision energy (Table 3). Figure 5A shows the MS^2 spectrum of the VP3 clip collected by fragmenting precursors from multiple charge states using a large isolation window (*m/z* 1,300–1,980, centered at *m/z* 1,640). By widening the isolation window, it is possible to co-isolate several charge states for one MS^2 scan. This is beneficial when not all charge states fragment equally well, and this option increases the chance of including a charge state that fragments well. Another benefit of such an approach is to increase the overall sensitivity by combining common fragments resulting from dissociation of different charge states of a protein. The desired number of charges defined by the AGC value can be accumulated more efficiently, supporting higher scan speed, which is beneficial for analyzing low abundant species and for experiments performed on an LC time scale.

All acquired MS^2 scans were analyzed by the top-down option in BioPharma Finder software using a database of all VP proteins. Usually, top-down fragmentation of the

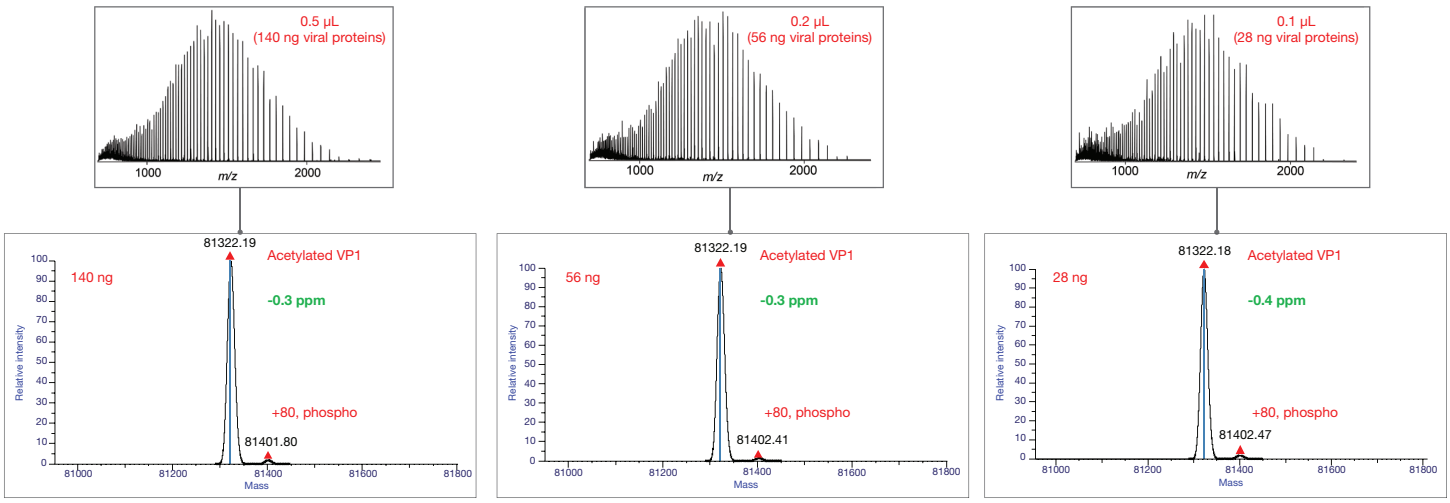


Figure 4. Full MS spectra representing an average of several scans acquired at 10 microscans each from VP1 (estimated total VP proteins on column: 140 ng, 56 ng and 28 ng, respectively) under denaturing conditions, acquired at a resolution setting of 15,000 (at *m/z* 200). The lower panel represents the deconvolution results. The mass accuracy was below 3 ppm for all injections.

proteins by HCD fragmentation provides fragment ion rich spectra with regions containing very highly charged fragment ions covering parts far into the protein sequence from either terminus. However as shown in Figure 5B for the precursor at *m/z* 1640 with RT around 17.5 min, only matching b-type ions were detected suggesting that the amino acid sequence of this precursor shares the same N-terminal as VP3. As determined via intact mass analysis, the average mass of this truncated form of VP3 was 43,221.32 Da, the potential amino acid sequence of the VP3 clip could be deduced by matching its average mass to the average mass of the C-terminal truncated VP3 sequence (Figure 5B, amino acid sequence highlighted in orange color corresponding to VP3 1–387 with acetylated N-terminus). To further confirm identity of the VP3 truncated form 1–387 (VP3 clip), we reanalyzed the MS^2 spectra using a VP3 1–387 sequence only database, and the proposed VP3 clip sequence was confirmed by a series of fragment ion matches from both N- and C- termini (Figure 5C). In addition, the described top-down approach was used to elucidate the sequence of the VP3 fragment eluted at RT 9.5 min, which was confirmed to be the truncated C-terminal sequence of VP3 (Figure 6).

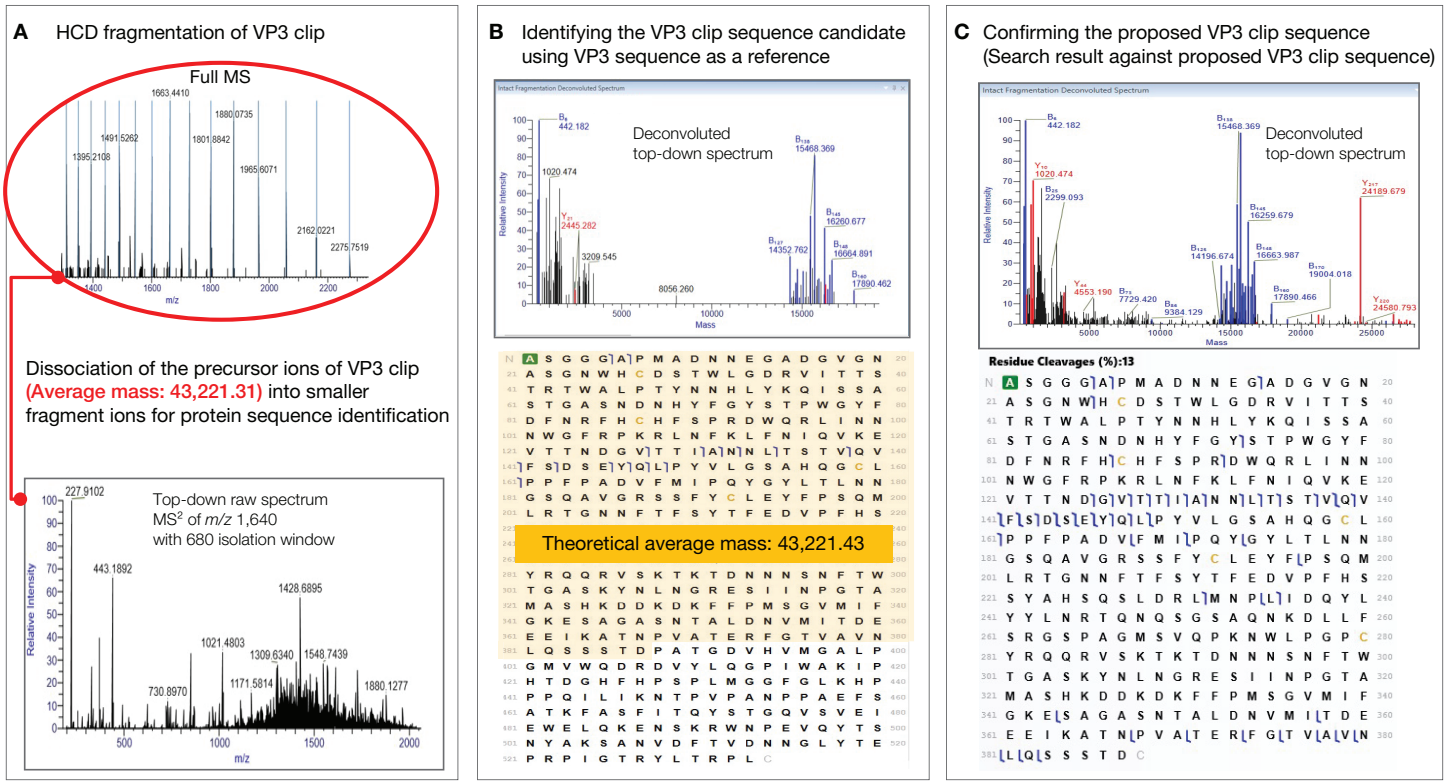


Figure 5. Intact/top-down analysis of VP3 clip. A) High-resolution average spectrum of VP3 clip and HCD fragmentation spectrum of *m/z* 1640 (+26) precursor. B) VP3 clip sequence candidate (highlighted in orange) deduced from the top-down fragment ion information and average mass measurement. C) The top-down data was searched against the proposed VP3 clip sequence (VP3 truncated form 1–387 with acetylated N-terminus) and confirmed the VP3 clip sequence by a series of fragment ion matches from both N- and C- termini.

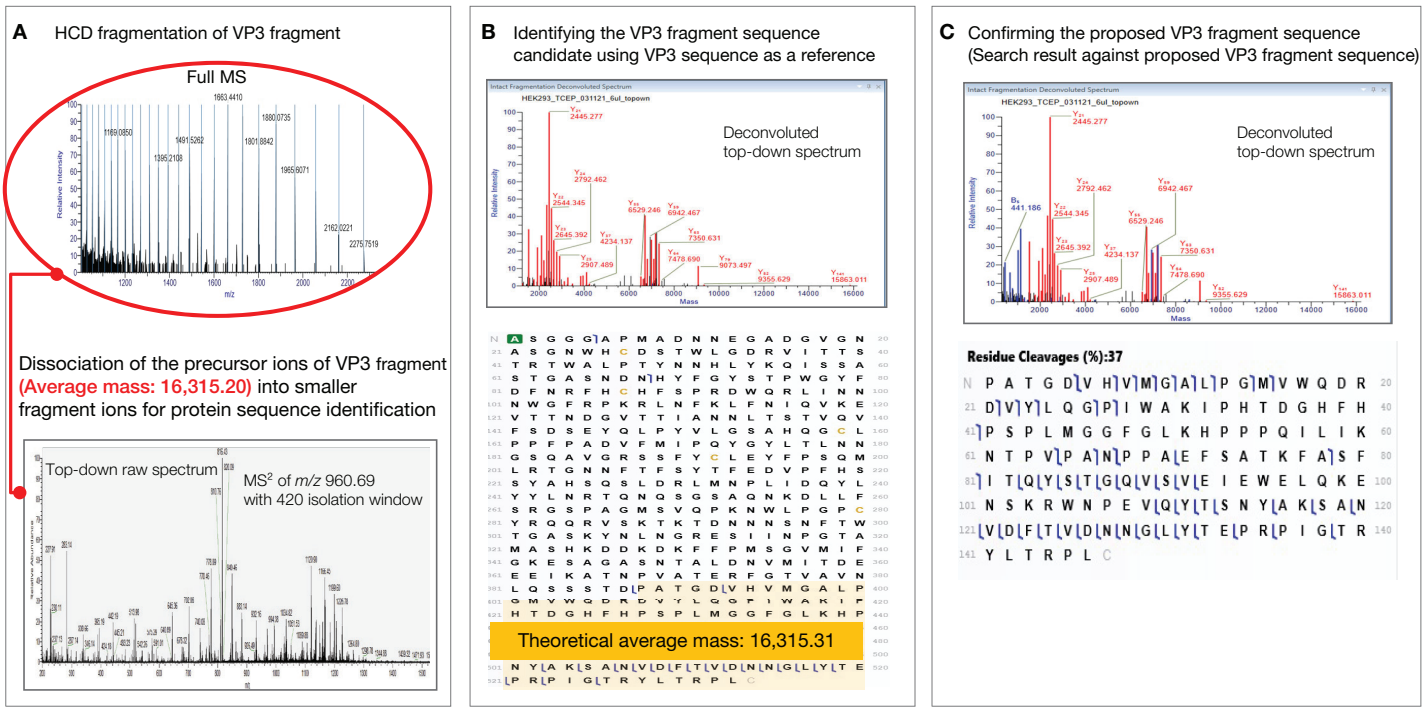


Figure 6. Intact/top-down analysis of VP3 truncated proteoform, average molecular mass 16,315.2. A) High-resolution average spectrum of truncated proteoform and OT HCD spectrum of *m/z* 960 (+17) precursor. B) VP3 truncated proteoform (highlighted in orange) deduced from the top-down fragment ion information and average mass measurement. C) The top-down data was searched against the proposed VP3 truncated proteoform sequence (truncated C-terminal sequence of VP3) and confirmed the VP3 truncated proteoform sequence by a series of fragment ion matches from both N- and C- termini.

Conclusion

In summary, the UHPLC-Orbitrap MS platform allows confident capsid protein characterization through intact and top-down mass analysis.

- High-quality MS and MS^2 data obtained for characterization of AAV proteins with great confidence.
- Excellent sensitivity and spectra quality of intact mass analysis enables accurate mass measurement of viral proteins and their low abundance variants.
- Unambiguous protein sequence confirmation of the low abundance truncated VP3 proteoforms using top-down approach.
- Dedicated software solution for both intact and top-down approaches.

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