# Characterization of Adeno-Associated Viral assemblies on an Ultra-High Mass Range Hybrid Quadrupole-Orbitrap Mass Spectrometer

Kristina Srzentić[1], Eugen Damoc[2], Marc Günder[1]
[1]Thermo Fisher Scientific, Reinach, Switzerland [2]Thermo Fisher Scientific, Bremen, Germany

## ABSTRACT

**Purpose:** Evaluate the performance of the Thermo Scientific™ Q Exactive™ UHMR hybrid quadrupole-Orbitrap™ mass spectrometer for intact mass analysis of viral assemblies under near native conditions and LC-MS intact and top-down mass spectrometry for AAV protein subunit analysis

**Methods:** (1) Intact mass analysis under native conditions using static nanospray, (2) Top-down analysis using RP LC-MS/MS

**Results:** Results demonstrate excellent performance of the Q Exactive UHMR mass spectrometer for analysis of AAV assemblies under near native and denaturing conditions.

## INTRODUCTION

Adeno-associated viral capsids (AAVs) are rapidly emerging delivery vehicles used to transport drugs into patients. In-depth knowledge about key structural characteristics of constituent capsid proteins, their proteoforms and their stoichiometries in viral assembly is critical for new modalities development in gene therapy and finding appropriate patient treatments. Mass spectrometry-based approaches can offer accurate intact mass determination and allows elucidation of intact proteoforms, afforded high resolution accurate mass measurement. In addition, analysis of product ions upon fragmentation at MS2 level can increase confidence in assigned proteoforms and oftentimes confirm their exact location within the amino acid sequence. Here we demonstrate the utility of an Orbitrap-based LC-MS platform to characterize the denatured AAV and their associated proteins with high sensitivity and mass accuracy.

## MATERIALS AND METHODS

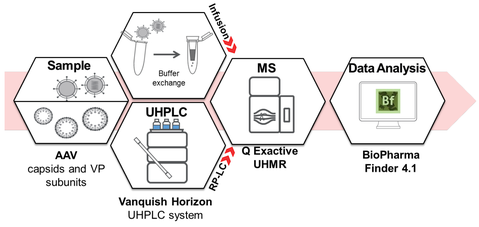
### Sample Preparation

AAV serotypes 2 and 6 were expressed and harvested in HEK293 cells. For intact mass measurement under near native conditions, AAV2 and AAV6 were buffer exchanged into 100 mM ammonium acetate using 30 kDa MWCO ( Millipore-Sigma). For capsid protein analysis, AAV6 was additionally incubated with acid to generate subunits.

### Liquid Chromatography and Mass Spectrometry

Intact mass analysis of viral assembly under near native conditions was performed by directly infusing the sample using coated emitters and static NanoFlex source. For intact capsid protein analysis under denatured conditions, and subsequent top-down analysis, RP LC-MS separation was accomplished using a Thermo Scientific™ Vanquish™ Horizon UHPLC system. For relative quantitation of empty vs full capsid, IEX separation was performed on a Thermo Scientific™ Vanquish™ Flex UHPLC system equipped with a Thermo Scientific™ Vanquish™ fluorescence detector. All intact MS and top-down MS measurements were carried out on a Q Exactive UHMR hybrid quadrupole-Orbitrap mass spectrometer.

**Figure 1.** General workflow for analysis of AAV viral assembly under near native conditions and analysis of AAV VP subunits analysis under denaturing conditions




### Data Analysis

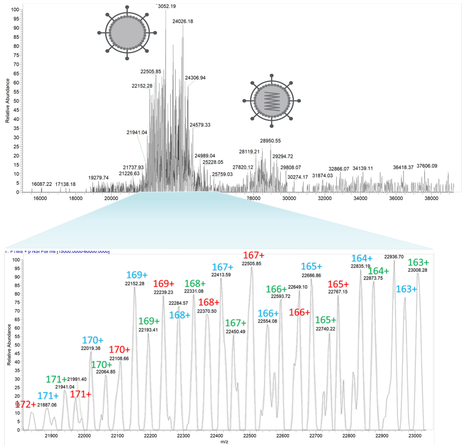
Thermo Scientific™ BioPharma Finder™ 4.1 software was used for intact deconvolution and top-down analysis

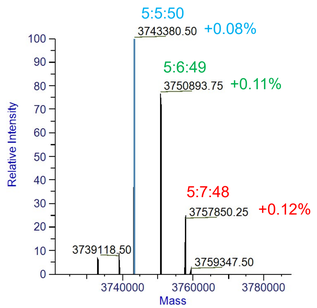
## RESULTS

### MS analysis of intact AAV2 under native conditions

Native MS analysis of intact AAV2 using static nanospray revealed the presence of at least three different forms with the following VP1:VP2:VP3 stoichiometries: 5:5:50, 5:6:49, and 5:7:48.

**Figure 2.** Native MS spectrum of AAV2 viral assembly showing baseline resolved 5:5:50, 5:6:49 and 5:7:48 forms

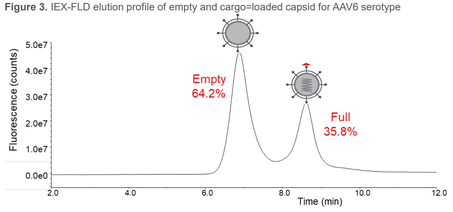



### IEX-FLD analysis of intact AAV6

IEX-FLD analysis of the intact AAV sample revealed the presence of empty and cargo-loaded capsid with a relative ratio for empty:full capsid of ~64% :36%.

**Figure 3.** IEX-FLD elution profile of empty and cargo=loaded capsid for AAV6 serotype




### MS analysis of AAV6 capsid proteins

RP LC-MS analysis of AAV6 serotype resulted in identification of several proteoforms of viral particle (VP) subunits comprised of truncated species in combination with a variety of post-translational modifications (PTMs), such as N-terminal acetylation and phosphorylation at serine (S) and threonine (T) sites in VP2. Integrated peak areas from total ion chromatogram (TIC) were used for relative quantitation. Relative abundances of the detected components were calculated against the integrated peak area of VP3, assigned as 100%. Determined ratio between detected subunits, VP1, VP2 and VP3 was 7:5:48, respectively.

**Figure 4.** RP LC-MS chromatogram

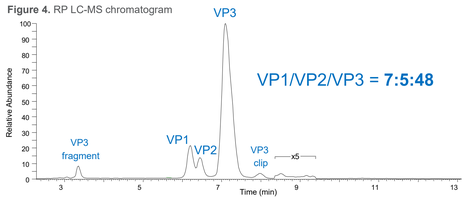
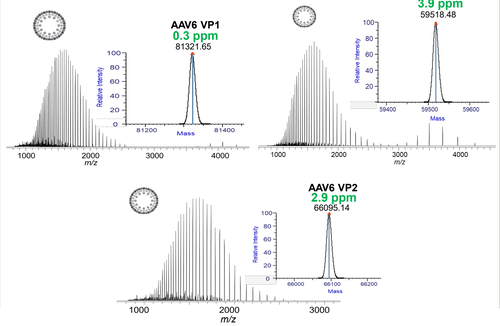



**Figure 5.** MS spectra of VP1, VP2 and VP3 subunits of AAV6




### Top-Down analysis of VP2 from AAV6

Top-down analysis under denaturing conditions was performed using All Ion Fragmentation (AIF) measurements on the Q Exactive UHMR instrument. Deploying AIF allowed for selection of entire charge state envelope of all major subunits, as well as truncated species, clip and proteoforms of VPs, thus enhancing fragmentation efficiency using HCD. Absolute collisional energy was set to 18, 20 and 22V in three separate acquisitions. The resulting LC-MS/MS spectra were searched with BioPharma Finder 4.1 and fragment ions matched using 10 ppm mass accuracy tolerance. In addition, HCD experiments resulted in well sequenced termini for all species. Presence of Ser-11 and Thr-56 phosphorylations in VP2 was confirmed by series of *b*-ions (see Figures 7A and 7B).

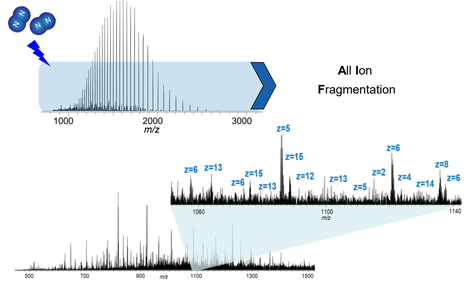
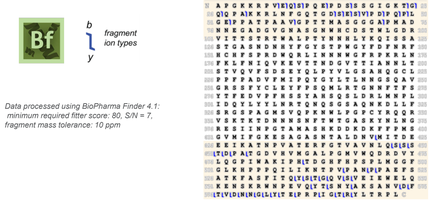
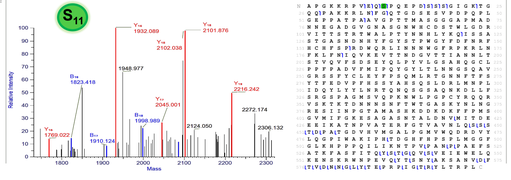



**Figure 6.** Graphical fragments map of VP2

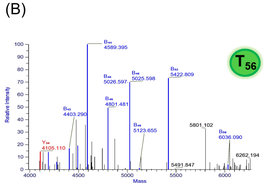



*Data processed using BioPharma Finder 4.1: minimum required filter score: 80, S/N = 7, fragment mass tolerance: 10 ppm*

**Figure 7.** AAV6 VP2 phosphorylation sites mapping using Top-Down analysis. (A )Phosphorylation of Ser-11, (B) Phosphorylation of Thr-56




(B)




## CONCLUSIONS

- Intact mass measurement under near-native conditions revealed presence of empty and cargo-loaded capsid in approximate ratio of 1:5.
- For the AAV2 we determined the presence of at least three different forms with the following VP1:VP2:VP3 stoichiometries: 5:5:50, 5:6:49, and 5:7:48.
- Time-resolved analysis of AAV6 serotype resulted in identification of several proteoforms of viral particle (VP) subunits comprised of truncated species in combination with a variety of post-translational modifications (PTMs), such as N-terminal acetylation and phosphorylation at serine (S) and threonine (T) sites in VP2.
- Intact mass analysis of VP subunits using 16 ms transient allowed mass determination with mass accuracy below 4 ppm.
- N-terminal acetylation of VP1 and VP3 subunits was directly confirmed from intact mass measurement due to excellent mass accuracy and spectral clarity obtained on Orbitrap-based mass spectrometer.
- Presence of phosphorylation PTM in VP2 was confirmed for T56 by series of *b*-ions, and in case S11 of also localized by adjacent $b_{12}$-ion.

## ACKNOWLEDGEMENTS

Authors would like to thank Julia Baek, Katy Irvin and Steven Milian for providing AAV samples, and wide team at Thermo Fisher Scientific who helped with method developments.



PO66125 EN0921S