QA/QC Analytical Methods for the Structural Analysis of Biotherapeutic Viruses

Estee Naggar Toole1,2, Craig Dufresne1, Alexander Schwann3, Ken Cook4, and Alexander R. Ivanov2*

1Thermo Fisher Scientific, West Palm Beach Fl, 33407; 2Barnett Institute of Chemical and Biological Analysis, Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115, United States3Thermo Fisher Scientific, Neuhofstrasse 11, 4153 Reinach, Switzerland 4Thermo Fisher Scientific, Stafford House, 1 Boundary Park, Hemel Hempstead, HP2 7GE, United Kingdom

Abstract

Gene therapy is a key treatment strategy for disorders caused by a missing or faulty gene and may involve addition, inhibition, editing, or functional replacement of a gene. Adeno Associated Viruses (AAVs) are common gene therapy vectors due to its high safety and effectiveness in delivering the Gene of Interest and its flexibility in targeting different tissue types.

Methods: This work focuses on establishing a set of analytical methods that can be used to validate structural features of Viral Capsid essential for efficacy as a biotherapeutic.

Results: Best fit sample preparation, column and instruments were selected to validate each of these essential AAV structural features.

Introduction

Adeno Associated Viruses (AAVs) are commonly utilized gene therapy viral vectors due to their safety and their flexibility in target tissue. It is essential to validate protein sequence, protein modification and/or encapsulation of genetic material to assure expected dosage and therapeutic benefit.(1,2,3) As with all biotherapeutics, any change in the biological structure could result in a loss or change in efficacy. The isohedral capsid of AAV is composed of 60 monomer capsid proteins in a 1:1:10 ratio molecular mass of approximately 87 kDa, 72 kDa, 62 kDa and an intact mass of 3746 kDa.(4,5) All viral capsid proteins (VP) have conserved sequences equivalent to VP3 with Nt extensions. Different serotypes have variable regions in the receptor binding regions which determine their target tissue types. Serotypes have sequence conservation in DNA interaction regions. Structural integrity of AAV viral capsid particles essential to ensure accurate dosing, appropriate function, and target cell targeting. (6,7) Serotypes are known to denature at distinct melting temperatures or below a pH of 4. (8,9)

As with all biotherapeutics, any change in the biological structure could result in a loss or change in efficacy. It is essential to validate protein sequence, protein modification, encapsulation of genetic material and purity of encapsulated genetic material to assure expected dosage and therapeutic benefit.

Viral capsids must be handled carefully as common sample preparation approaches can result in sample loss to aggregation and precipitation. Here we explore and compare the ability of different separation strategies and stationary phases to evaluate the purity and structural integrity of biotherapeutic materials.

It is essential to validate protein sequence, protein modification, viral capsid occupancy and purity of encapsulated genetic material to assure expected dosage and therapeutic benefit. Orthogonal chromatographic approaches are discussed here to validate these capsid components.
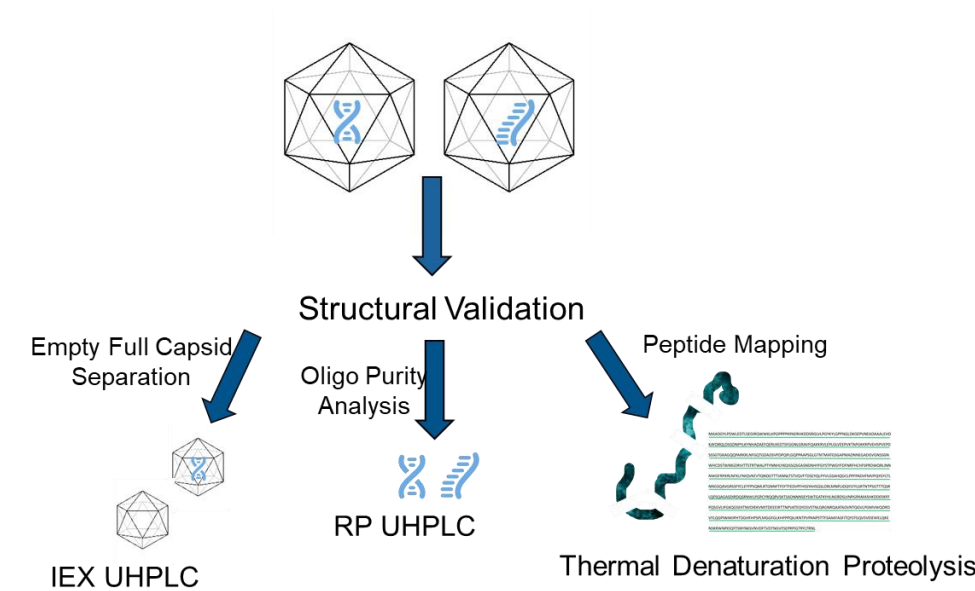
Materials and methods

Sample Preparation

Samples were prepared using the AAV-MAX Transfection Kit (A50515)

Thermal Denaturation Proteolysis was conducted using SMART Digest™ Pepsin Kits (60109-110)

Test Method(s)

Oligo separations were performed using DNAPacTM RP2.1 x 100 mm, 4 µm (088923)

Peptic Digest product peptides were resolved using EASY-Spray™ HPLC Columns 75 µmx500 mm, 2 µm (ES903)

Empty and Full capsid separations was achieved using ProPacTM SAX-10, 2 x 50 mm

Data Analysis

Chromeleon™ Chromatography Data System (CDS) Software (CHROMELEON7)

BioPharma Finder™ Software (OPTON-30986)

Instrumentation

Vanquish™ Horizon UHPLC System (IQLAAAGABHFAPUMZZZ)

Vanquish™ Neo UHPLC System (VN-S10-A-01)

Orbitrap Exploris™ 480 Mass Spectrometer

Results

Described here are sample preparation, column, instruments and their conditions selected to validate each essential AAV structural features. Protein sequence, protein modification, viral capsid occupancy and purity of encapsulated genetic material to assure expected dosage and therapeutic benefit. Preparative and chromatographic approaches are discussed here to validate these capsid components.
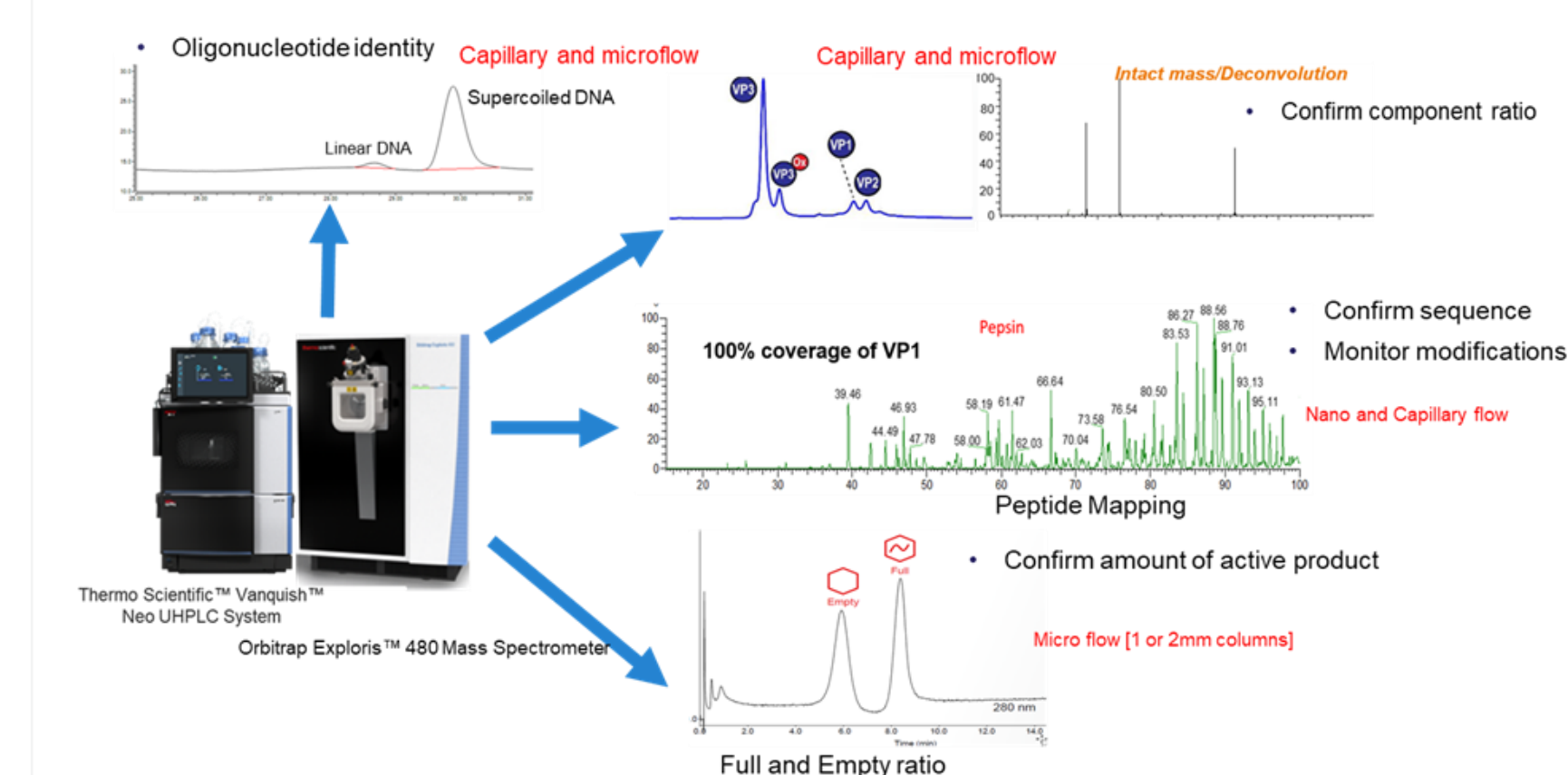

Figure 1: Features essential to the structural integrity and their purity in bioactive viral vector therapeutics can be validated by parallel reversed phase and ion exchange chromatographies using UV and/or mass spectrometry. The combination of these methods can establish a means to establish a fast and reproducible protocol.
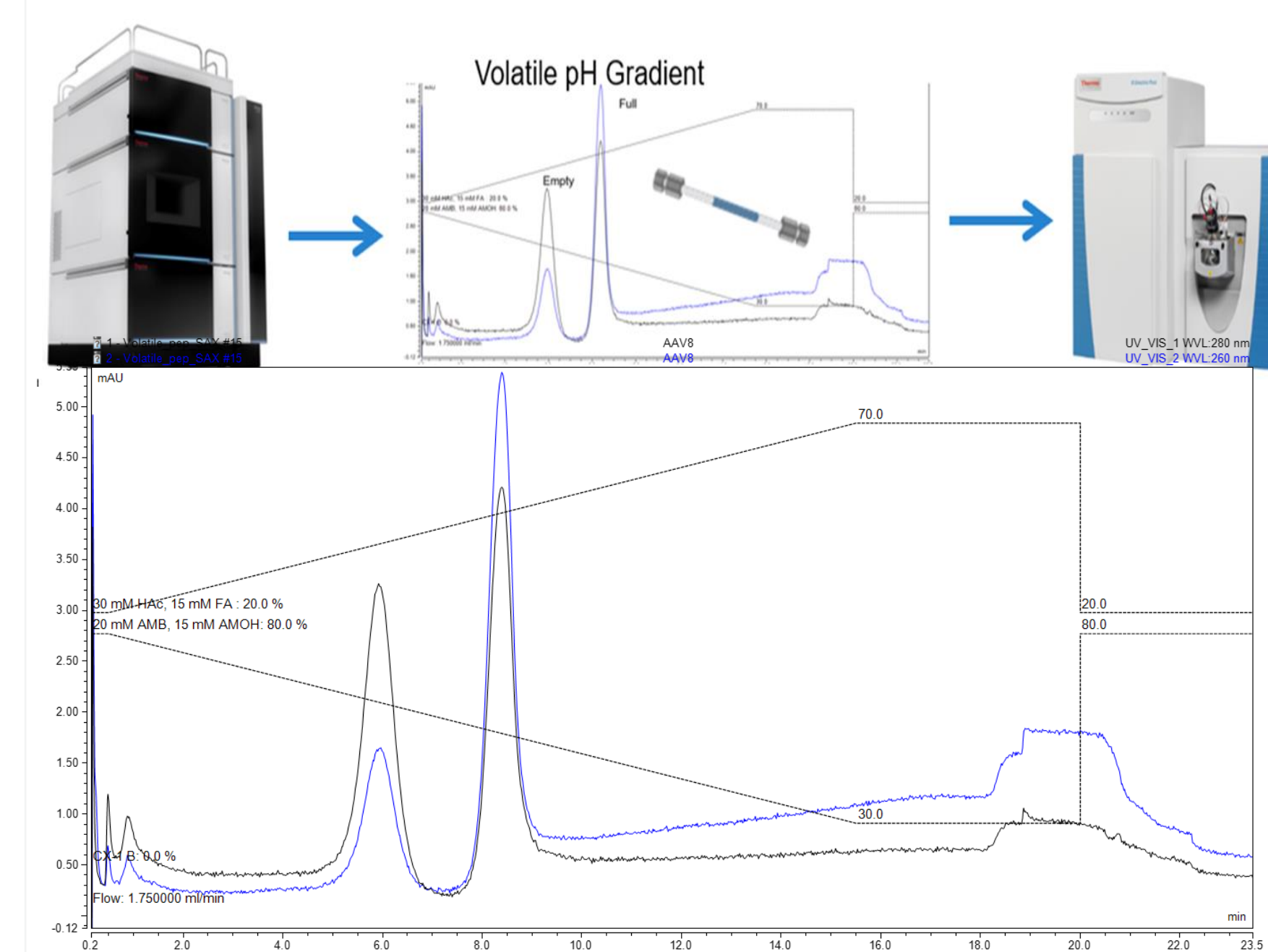

Figure 2. Empty/Full Viral Capsid separation can be achieved using the ProPac SAX 2 x 50mm at a temperature of 30C monitored at the wavelengths 260 nm and 280 nm. This separation entails a pH gradient with Buffer A = 20mM ammonium Carbonate, 0.395g 15mM Ammonium hydroxide 258ul and Buffer B = 15mM Formic acid 149ul in 250ml 30mM Acetic acid 431ul
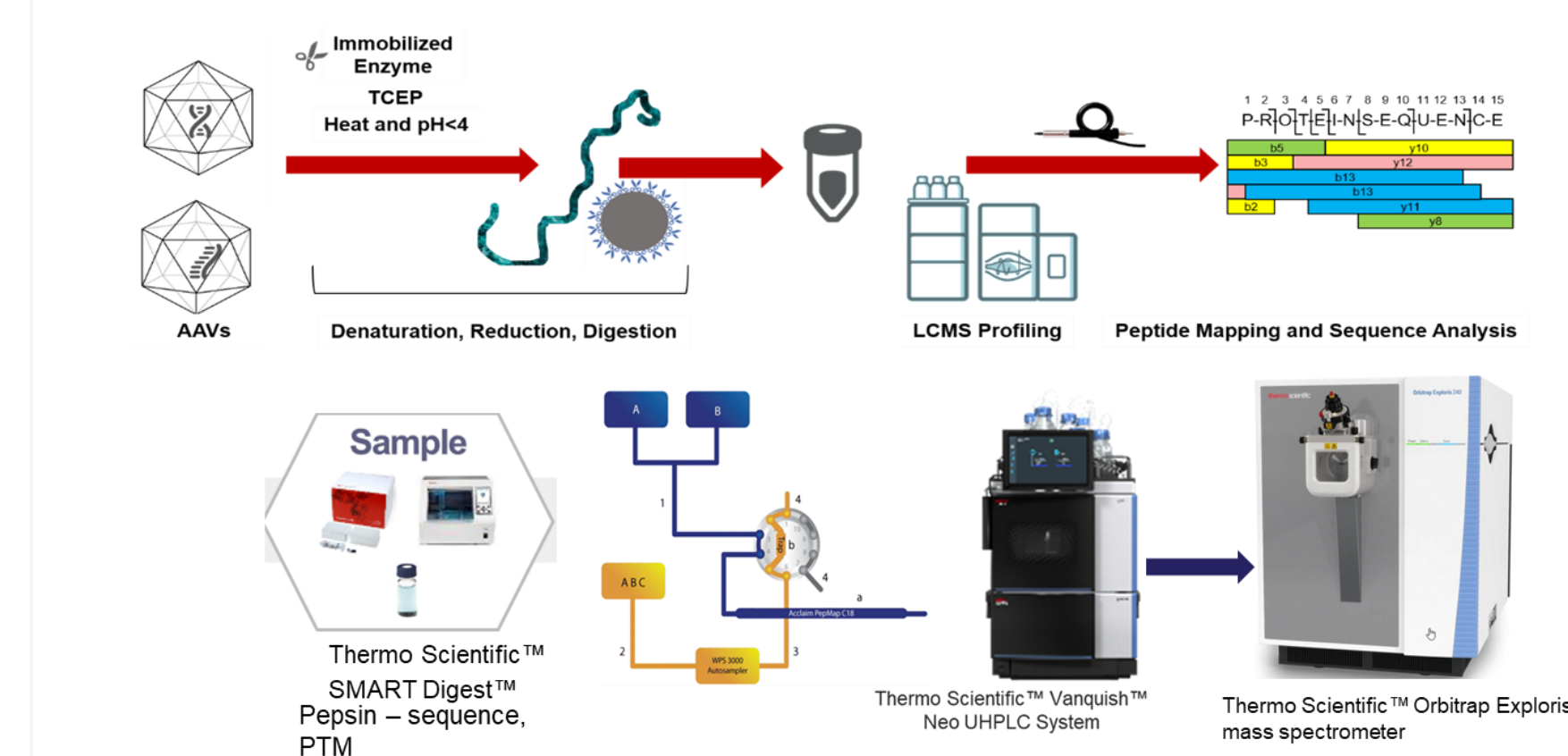

Figure 3. SMART proteolysis protocol is shorter, fast and reproducible. It entails a single enzyme digestion including prepared digestion buffer, (optionally) reducing agent TCEP, and beads with immobilized enzyme to ng amount of sample, followed by heating and shaking for up to 60 min. The digestion is quenched by removing the enzyme tethered beads and samples are ready for direct injection and analysis by NanoLC MS. This allows the protocol to be completed in half a day. There are no steps that will deplete concentration of sample and 100% sequence coverage can be obtained with a single enzyme.
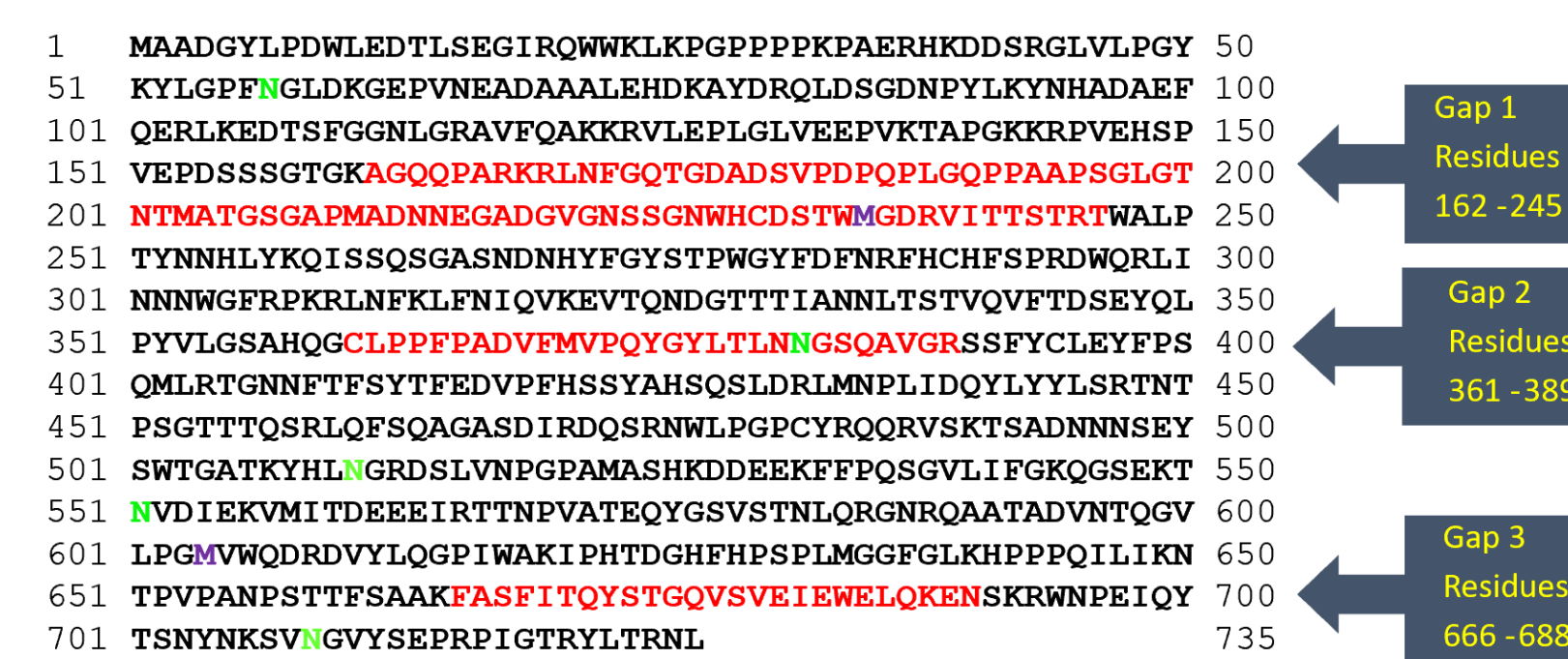

Figure 4: AAV2 contains 4NG sites, shown in green, which are known to readily undergo deamidation. Note one NG site is localized in a region that lacks sequence coverage in the tryptic peptide map.
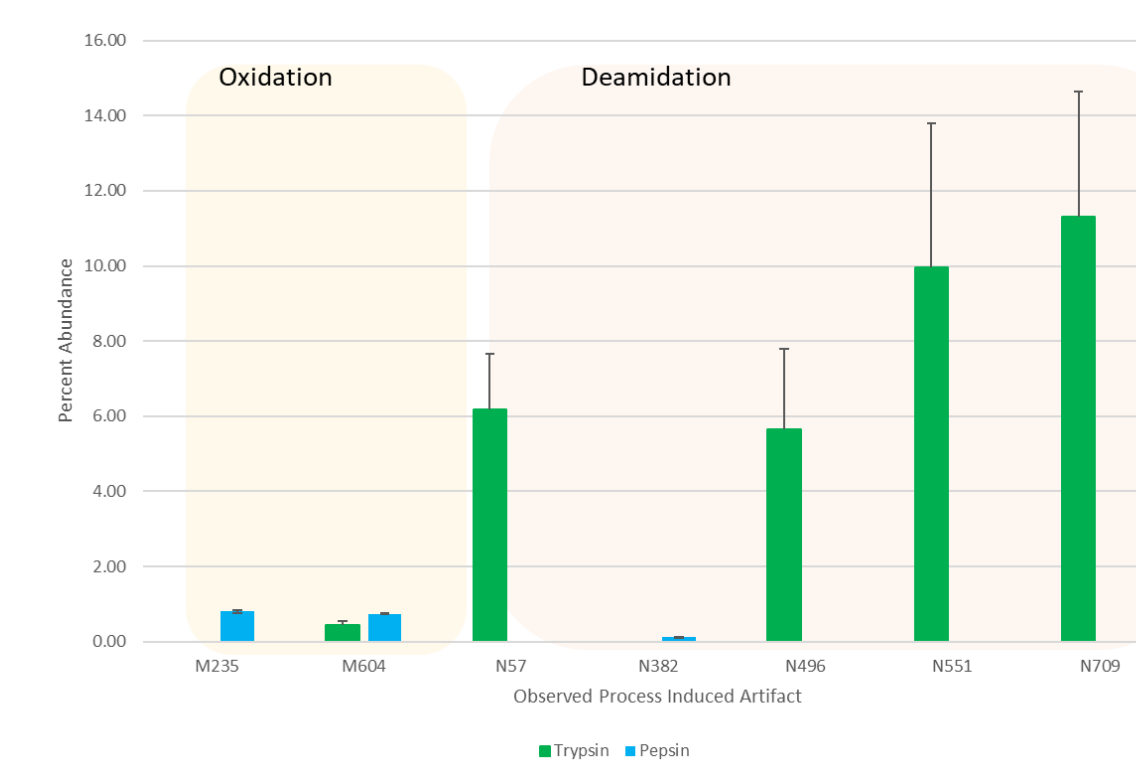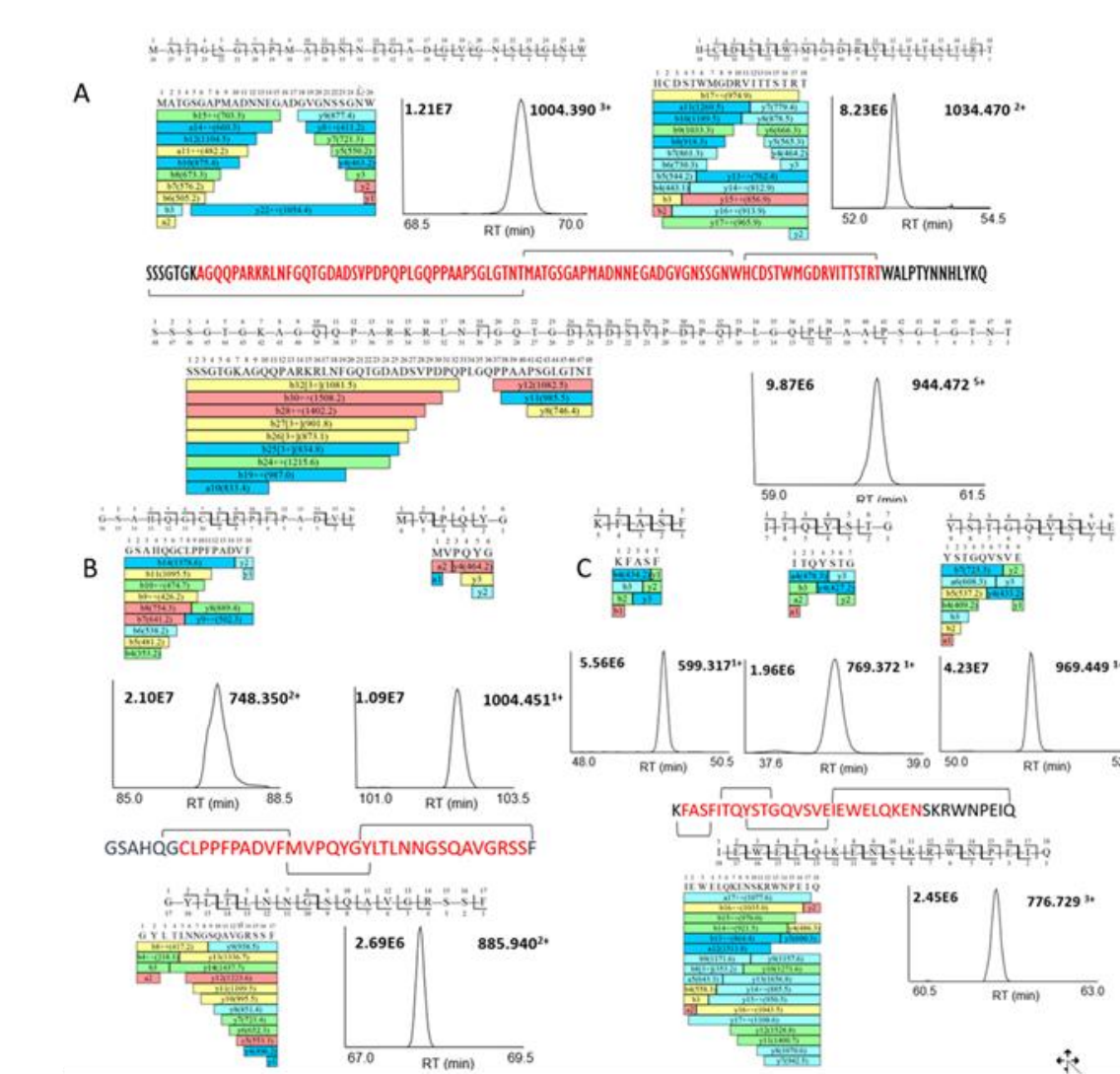

Figure 5. Looking at asparagine deamidation across these NG sites and at one NV site, we see that deamidation levels of the tryptic digests range from 6-11% whereas they are negligible in the peptic digest products which is to be expected as the peptic digestion is performed at a pH of 3.5, vs 6.8 of the tryptic digestion.

Figure 6: Pepsin products are comprised of smaller overlapping peptides due to the lower specificity of the enzyme as compared with Trypsin. Peptic peptides are heterogeneous and overlap to provide sequence coverage in viral protein areas where tryptic peptides leave gaps. Shown here are representative peptides that fill in tryptic coverage gaps. Digest products are high intensity, show symmetrical peak shape in their EIC and have high confidence fragmentation patterns
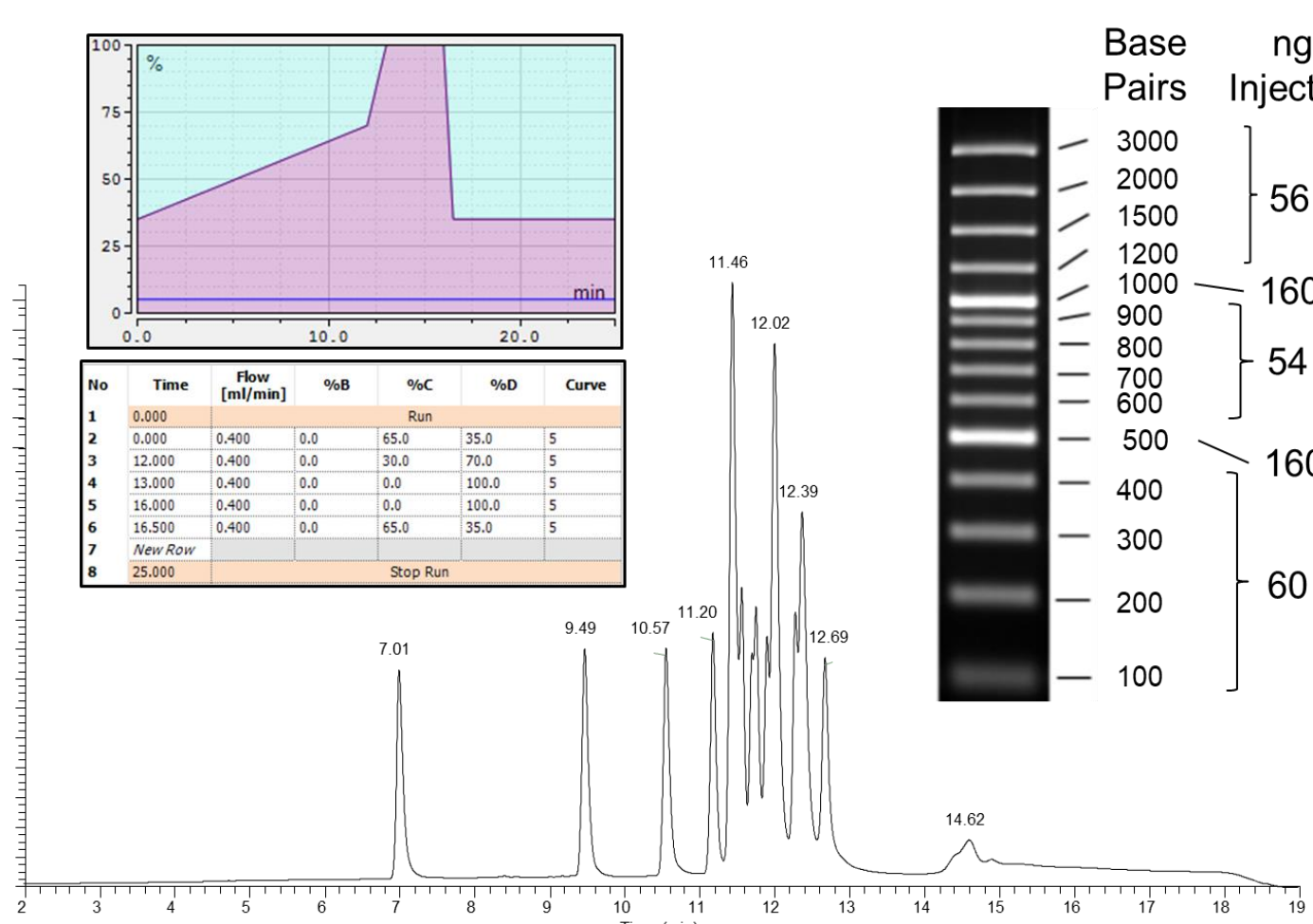
Figure 7: Encapsulated genetic material can be analyzed using a DNAPacTM RP 088923 2.1 x 100 mm, 4 µm at a temperature of 60⁰ C with preheating, 100 um ViperTM tubing monitored at UV 260 nm. Mobile phases :
A: 0.1 M Triethyl ammonium acetate (TEAA)
B: 0.1 M Triethyl ammonium acetate (TEAA) in 25% Acetonitrile were used for this separation. Gradient can be adjusted for optimal resolution and desired run time.

Conclusions

- Optimal sequence coverage can be achieved using heat and pH as a denaturant and Pepsin as the proteolytic enzyme
  - Digest product of heat stable pepsin provide a more complete protein sequence coverage than products of heat stable trypsin
  - Digest products of heat stable pepsin are smaller and more sequence diverse than those of heat stable trypsin. They are also comprised of smaller overlapping sequence segments and have less specificity as to C terminus residue
  - Digest products of pepsin contain negligible levels of deamidation as compared to 6-11% deamidation in tryptic digest products
  - Increased chromatographic efficiency in peptic digest products contributes to increase in sequence coverage

- Baseline separation of full and empty capsid can be achieved using a pH gradient with the the ProPac SAX 2 x 50mm
  - A survey scan must first be produced then the gradient can be adjusted for a shorter run time
  - pH gradients are a fast and efficient means to develop a serotype specific methods
- .Encapsulated genetic material can be analyzed using a DNAPacTM RP 088923 2.1 x 100 mm
  - This can be used to profile or evaluate purity of encapsulated material

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