Detection of AAV structure: capsid proteins and nucleic acid composition on LabChip® GXII Touch™ Platform

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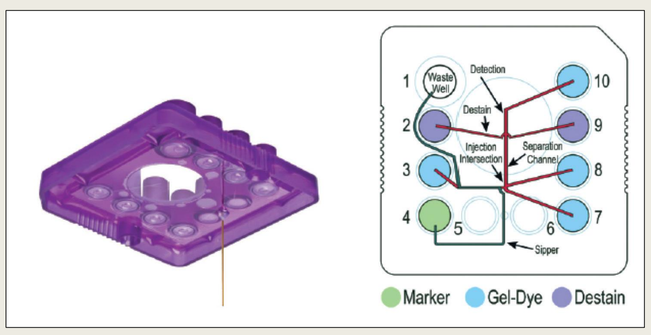
1 Introduction

For over fifty years, adeno-associated virus (AAV) has remained an elusive object in virology studies and gene therapeutics due to its non-pathogenicity. Many therapeutic companies are currently involved in recombinant AAV production with needs of viral particle characterization in terms of capsid structure and nucleic acid content to estimate therapeutic doses.
AAV is a protein shell encapsulating a single-stranded DNA genome of 4.8 kb, which is dependent on co-infection with other viruses to replicate. Its genome contains *Rep (*Replication), *Cap (*Capsid), and Assembly genes. The *Rep* gene encodes four proteins, which are required for genome replication and packaging, while *Cap* gene produces capsid proteins (VP1/VP2/VP3) to form protective outer shell and to perform cell binding. All VPs are the product of the same genome which becomes evolutionally modified to form a more rigid capsid structure and gain extra functionality[1].
The PerkinElmer LabChip® GXII Touch™ HT platform is an instrument to perform protein and nucleic acid analysis with high sensitivity and reproducibility. The instrument can distinguish differences of protein molecular weight as low as 1kDa, which can be crucial to characterize recombinant capsid proteins.
Additionally, the fast sample run times from multi-well plates allow for high throughput experiments and can open opportunities to monitor kinetic processes such as breakage of virus particles or their interactions with receptors.

2 ProteinEXact™ Assay to evaluate AAV capsid proteins

LabChip® GXII Touch™ Instrument



Sipper Chip in Quartz

Microfluidic Channels

Because of the increased interest in AAVs in gene therapies, there exists a need for a method to properly characterize AAV capsid proteins (VPs) to accurately benchmark viral infectivity and potency.
PerkinElmer's ProteinEXact™ assay for the LabChip® GXII Touch™ protein characterization system utilizes IntelliChip™ assay optimization technology to enable high resolution and reproducibility of protein concentration analysis of a sample to enable the utmost of accuracy in AAV characterization for recombinant viral production.
AAVs vary in stability (temperature at which capsid breaks) and have a similar ratio of capsid protein composition known as VP1:VP2:VP3 = 1:1:10[2]. The ProteinEXact™ kit can be used to confirm this property of recombinant viruses to conserve optimum infectivity. Empty capsid particles of several serotypes (AAV2, AAV8 and AAV9) have been used for these experiments. To break apart capsid particles, AAV samples were repeatedly heated in the presence of detergents. As different AAVs have varying stability, the temperatures were individually chosen for each serotype. As shown in Figure 1, fully broken AAV8 particles have only three peaks with sizes of approximately 80, 90 and 115kDa molecular weights.

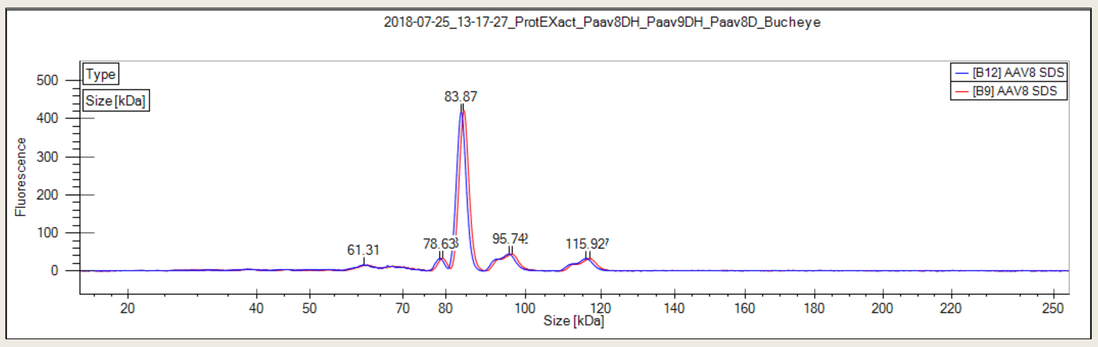
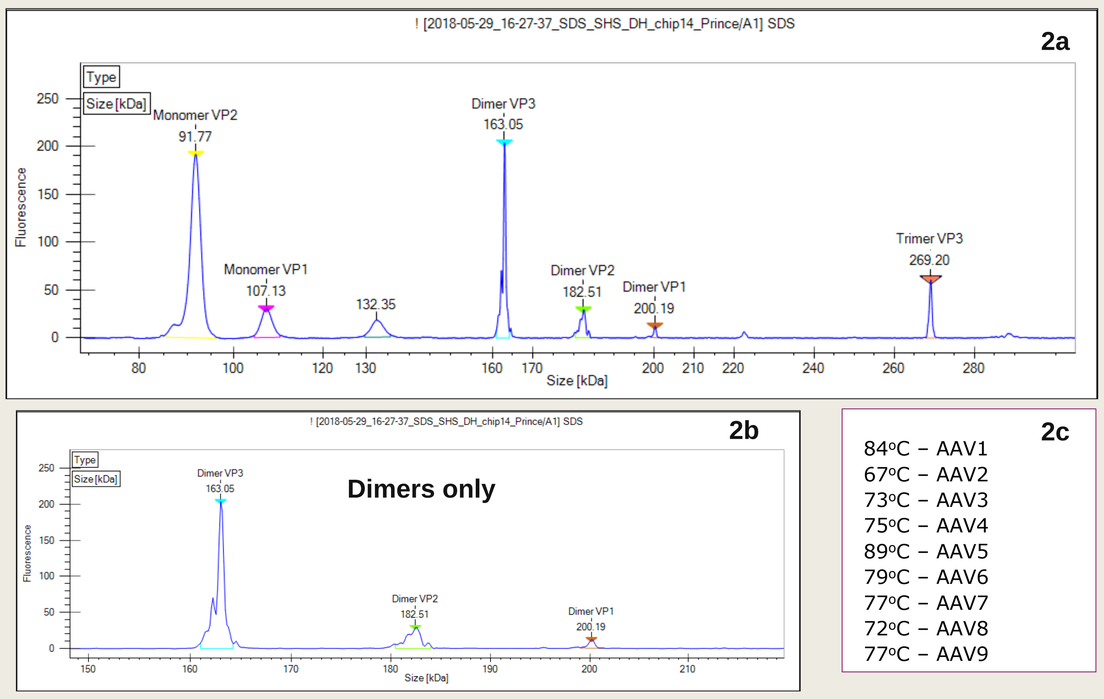


Figure 1. Profiles of capsid proteins after full breakage of AAV8 particles. Two run overlay shown to demonstrate reproducibility

3 Observation of AAV kinetics (Stability)

To investigate the time scales of AAV breakage, kinetic experiments have been performed with each recombinant construct. The LabChip® GXII Touch™ HT protein characterization system is able to collect up to 300 experimental points every 30-50 seconds, which gives additional opportunity to investigate the full kinetics of capsid breakage.



2c

84°C – AAV1
67°C – AAV2
73°C – AAV3
75°C – AAV4
89°C – AAV5
79°C – AAV6
77°C – AAV7
72°C – AAV8
77°C – AAV9

Figure 2a & 2b. Partially broken AAV8 particles (b-dimers only) during kinetic experiments.

Figure 2c – Stability of AAVs with different serotypes.

Partially broken capsids can consist of a mixture of different protein molecular weights (monomers, dimers, trimers, etc.) as seen in Figure 2 as well as non-broken particles, which have sizes of approximately 25 nm[3].

The latter species should be avoided to prevent possible clogging of LabChip® system channels during an experiment. 96-well plates with AAV samples in the presence of surfactants were heated to individual temperatures and immediately loaded into the instrument.
The electropherogram in Figure 2a demonstrates the presence of both dimers and trimers; characteristic of partially broken viral particles. The disappearance of dimer/trimer peaks later can demonstrate the end of breaking kinetics (not shown).
Individual protocols were developed for AAV capsid particles with various melting temperatures (Figure 2c)

4 AAV Glycosylation

Some investigators observed well-distinguished "shoulders" on the main VP3 peaks during experiments on LabChip® GXII Touch™ protein characterization system.
Recently, Sara Murrey et.al.[4] reported certain glycosylation of AAV2 capsid proteins and theoretically estimated the ability of capsid protein surfaces to have four possible locations for glycosylation.
To investigate this hypothesis of capsid glycosylation, we performed de-glycosylation of AAV2, AAV8 and AAV9 particles using enzymatic treatment (digestion by PNGase F), previously developed in our R&D laboratory; the data in Figure 4 show AAV8 profiles before and after that treatment. In Figure 4, VP3 peaks were detected to have a shifted molecular weight of 79kDa, compared to 81kDa in the untreated VP3. The same change of about 2kDa can be observed for VP2 (92 vs. 94kDa) and VP1 (113 vs. 115kDa). The observation agrees with the average molecular weight of glycans attached to capsid proteins.
Additionally, each of the AAV dimers, shown in enlarged Figure 2b, have up to three shoulders, supporting the hypothesis of multiple glycosylation of viral proteins.

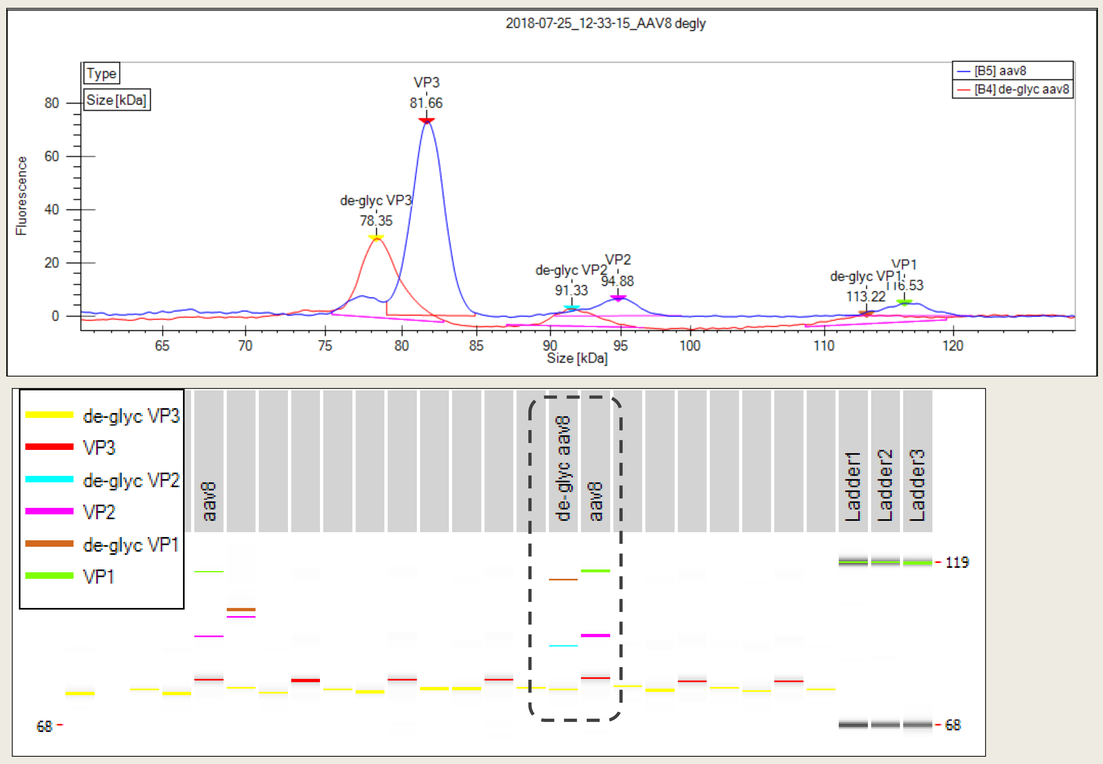


Figure 3. demonstrates overlay electropherograms with the same changes in molecular weights for all three capsid proteins. Also, well resolved shifts in molecular mass bands are shown on classical gel-like presentation of results which is also available on the LabChip® GXII Touch™ protein characterization system.

5 Detection of genomic DNA from AAV capsids

Additionally to accurate protein analysis, PerkinElmer's DNA assay for the LabChip® GXII Touch™ platform is enable to measure of genomic DNA concentration of broken viral sample with high resolution and reproducibility.
Established limit of detection of LabChip® GXII Touch™ for DNAs and proteins allows to estimate the *percentage of empty capsids* important for dose determination.

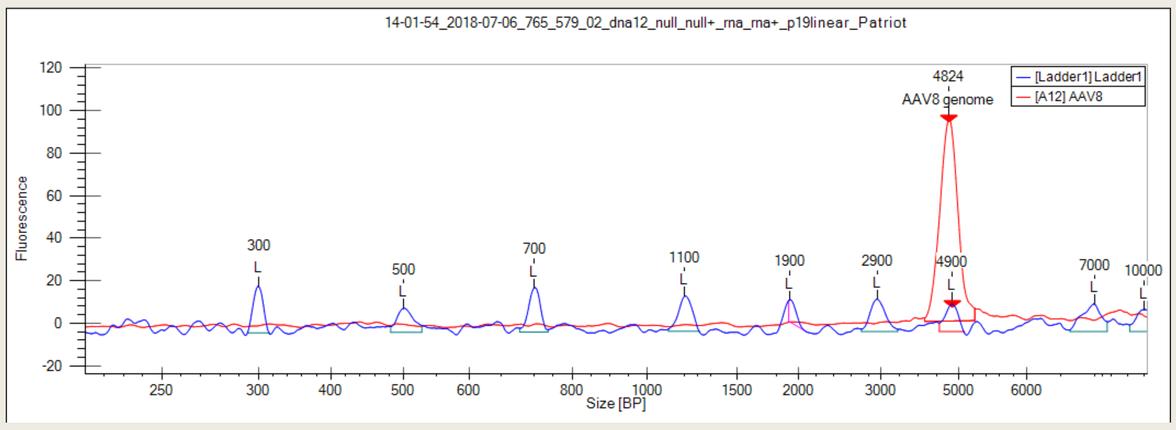


Figure 4. Profile of genomic DNA released from AAV8 viral particles after heating in the presence of surfactants.

6 Citations

1. Bennett, A. et.al. Thermal stability as a determinant of AAV serotype identity. 2017, Molecular therapy: methods and clinical development, 6:171-182.
2. Hermonat, P. L., and N. Muzyczka. Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells.1984 Proc. Natl. Acad. Sci. USA 81:6466-6470.
3. Xie, Q., et.al. The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. Proc. Natl. Acad. Sci. USA 2002 99:10405–10410.
4. Sara Murrey et.al. Characterization of the capsid protein glycosylation of adeno-associated virus type 2 by high-resolution mass spectrometry 2006 Journal of Virology 80: 6171–6176.

7 Summary

- Presented data on PerkinElmer's ProteinEXact™ assay for the LabChip® GXII Touch™ protein characterization system with AAV samples demonstrate the ability of the platform in providing a full analysis of viral proteins.
- The assay provides a robust method for serotype identification of clinical vectors.
- The highly reproducible and accurate measurements of capsid composition can be a novel method for the characterization of recombinant viral particles, a crucial step in gene therapy applications.
- High-resolution verification of capsid protein glycosylation further benefits production of therapeutically important components.

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