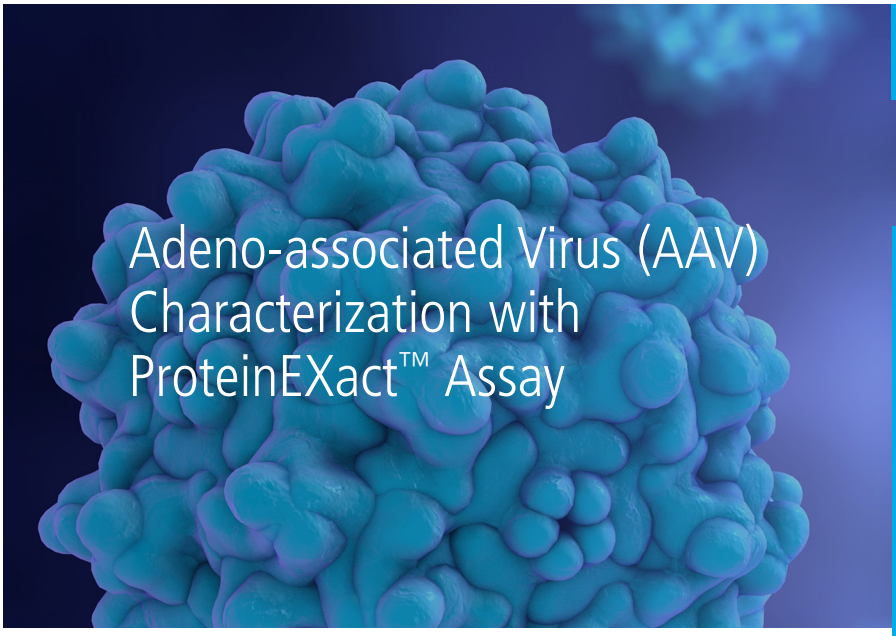

# Adeno-associated Virus (AAV) Characterization with ProteinEXact™ Assay

TECHNICAL NOTE

## LabChip® GXII Touch™ Protein Characterization System

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The discovery of DNA as the biomolecule of genetic inheritance has led to the concept of therapies in which mutant, damaged genes could be altered to improve the human condition. The ability to rapidly and affordably perform genetic testing on hundreds and thousands of samples, and to sequence complete genomes, has resulted in an exponential increase of information and has allowed for the identification of gene(s) which drive a disease state. Mutant gene(s) can then be potentially be 'fixed', or expression normalized if the disease could be treated or cured at the molecular level.

Several viral agents and artificial delivery technologies have been utilized in an attempt to safely deliver protein receptors or nucleic acid cargo into a host cell for replication to potentially reverse the disease state. However, undesired properties of some viral vectors, including their immunogenic profiles or their propensity to cause strong side effects or even cancer have resulted in serious clinical adverse events and limited current use in the clinic to certain applications.

Adeno-associated virus (AAV) is one of the most actively investigated gene therapy vehicles today due to its non-pathogenicity. AAV is a protein shell encapsulating a small, single-stranded DNA genome of approximately 4.8 kilobases (kb). AAV belongs to the parvovirus family and is dependent on co-infection with other viruses to replicate. Its single-stranded genome contains three genes: *Rep* (Replication), *Cap* (Capsid), and *aap* (Assembly). The Rep gene encodes four proteins (Rep78, Rep68, Rep52, and Rep40), which are required for viral genome replication and packaging, while *Cap* expression gives rise to the viral capsid proteins (VP; VP1/VP2/VP3), which form the outer capsid shell that protects the viral genome, as well as being actively involved in cell binding and internalization. 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].



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## Protein EXact™ Assay

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.

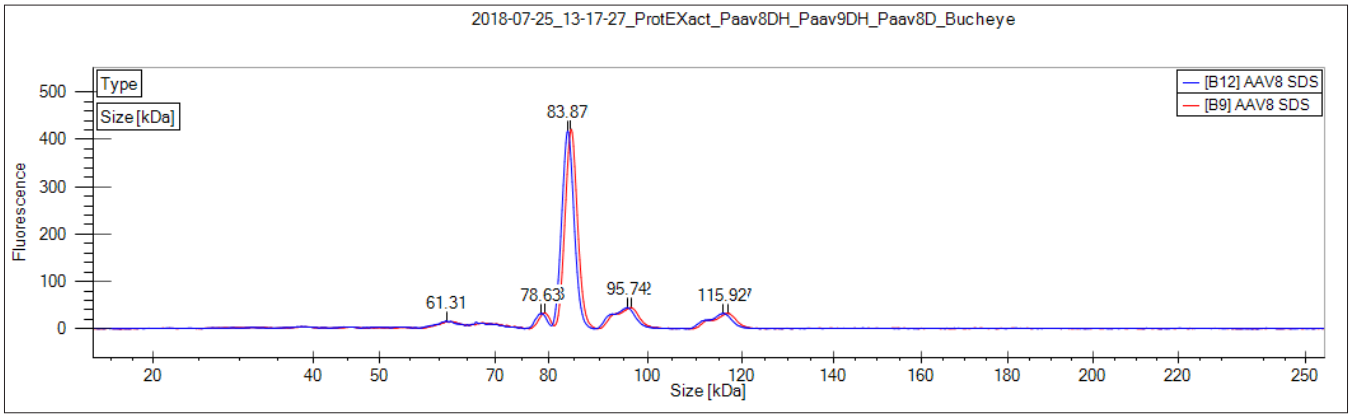


***Figure 1.*** Profiles of capsid proteins after full breakage of AAV8 particles. Two run overlay shown to demonstrate reproducibility.

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 115 kDa molecular weights after heating at 80°C in the presence of SDS.

### 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™ protein characterization system is able to collect up to 300 experimental points ever 30-50 seconds which can potentially provide additional insights on capsid breakage.

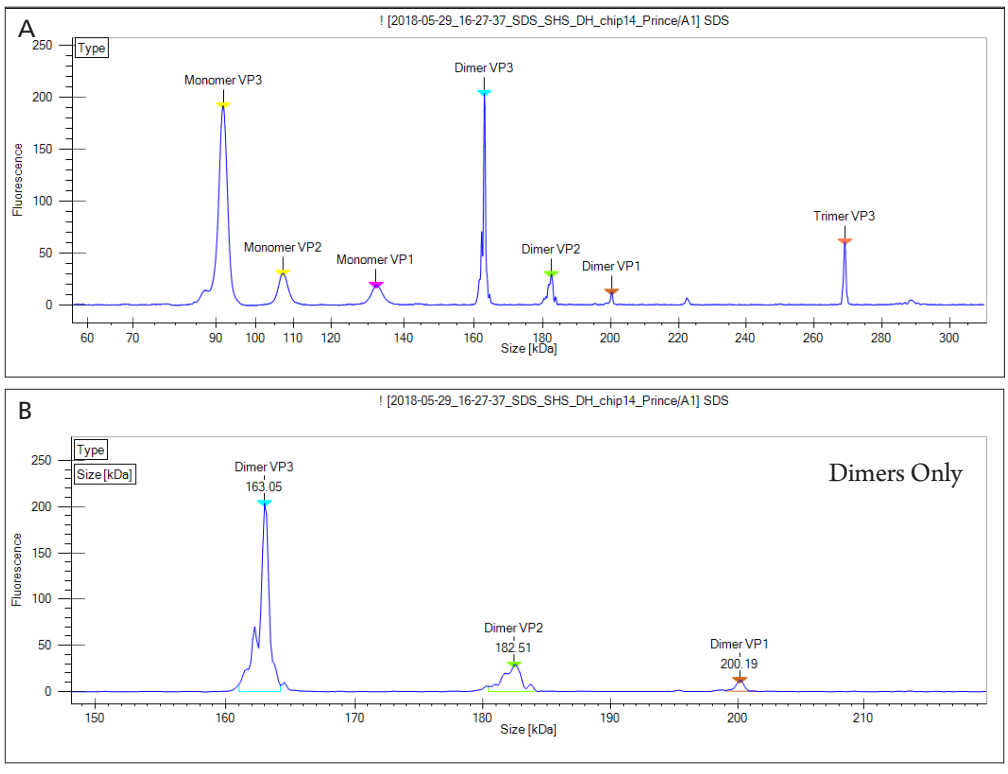


***Figure 2A & 2B.*** Partially broken AAV8 particles (b-dimers only) during kinetic experiment.

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 1% of SDS and TE buffer 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).

## 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 3 show AAV8 profiles before (a) and after (b) that treatment. In Figure 3b, VP3 peaks are observed to have a shifted molecular weight of 79 kDa, compared to 81 kDa in the untreated VP3 in Figure 3a. The same change of about 2 kDa can be observed for VP2 (92 vs. 94 kDa) and VP1 (113 vs. 115 kDa). The observation agrees with the average molecular weight of glycans attached to capsid proteins.

Additionally, each of the AAV dimers, shown in enlarged Figure2b, have up to three shoulders, supporting the hypothesis of multiple glycosylations of viral proteins.

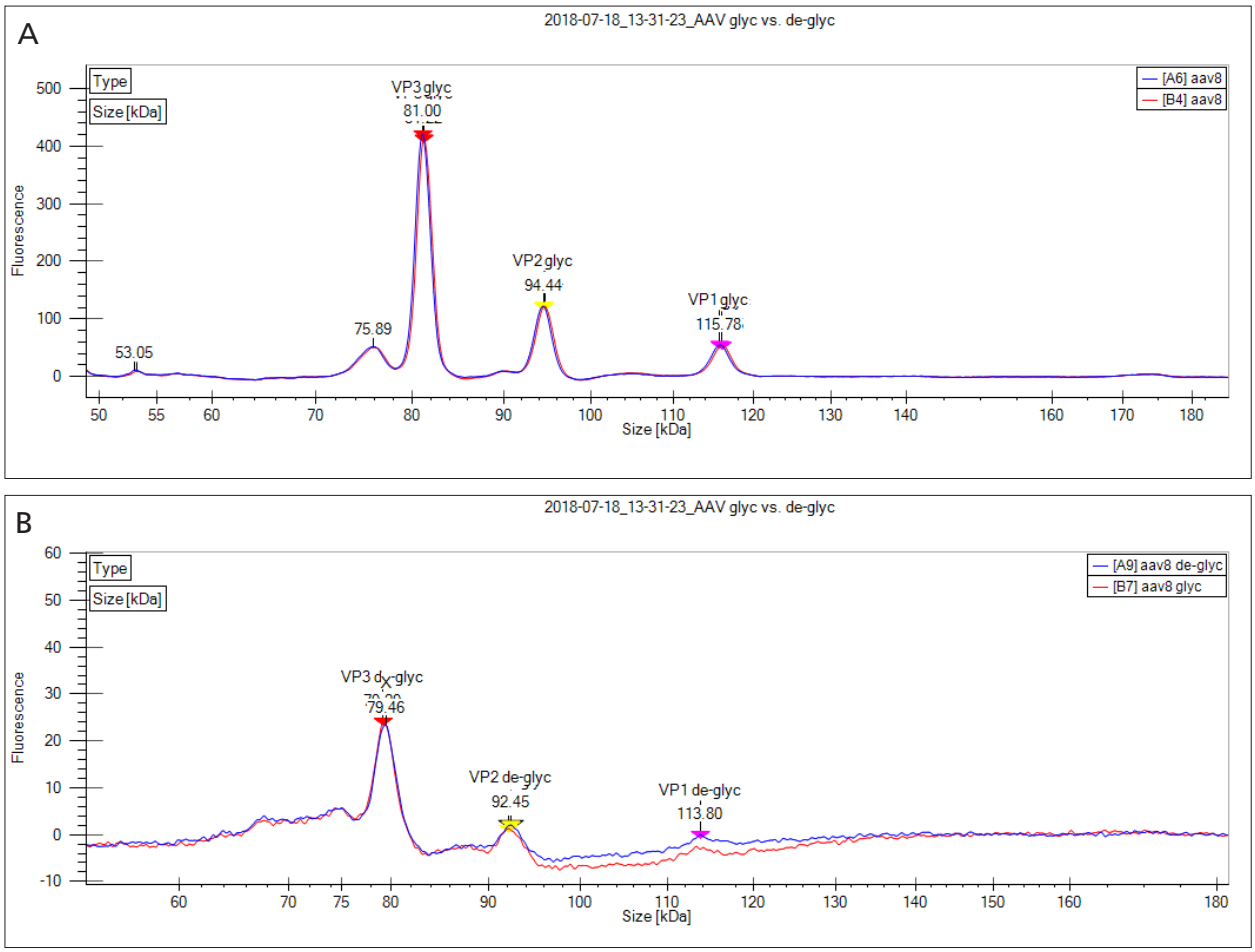


***Figure 3A & 3B.*** De-glycosylation results of broken AAV8 particles (experiment 1).

To prove reproducibility of de-glycosilation an additional experiment was performed with broken AAV particles. Figure 4 demonstrates overlay electropherograms with the same changes in molecular weights for all three capsid proteins as on Figure 3. 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.

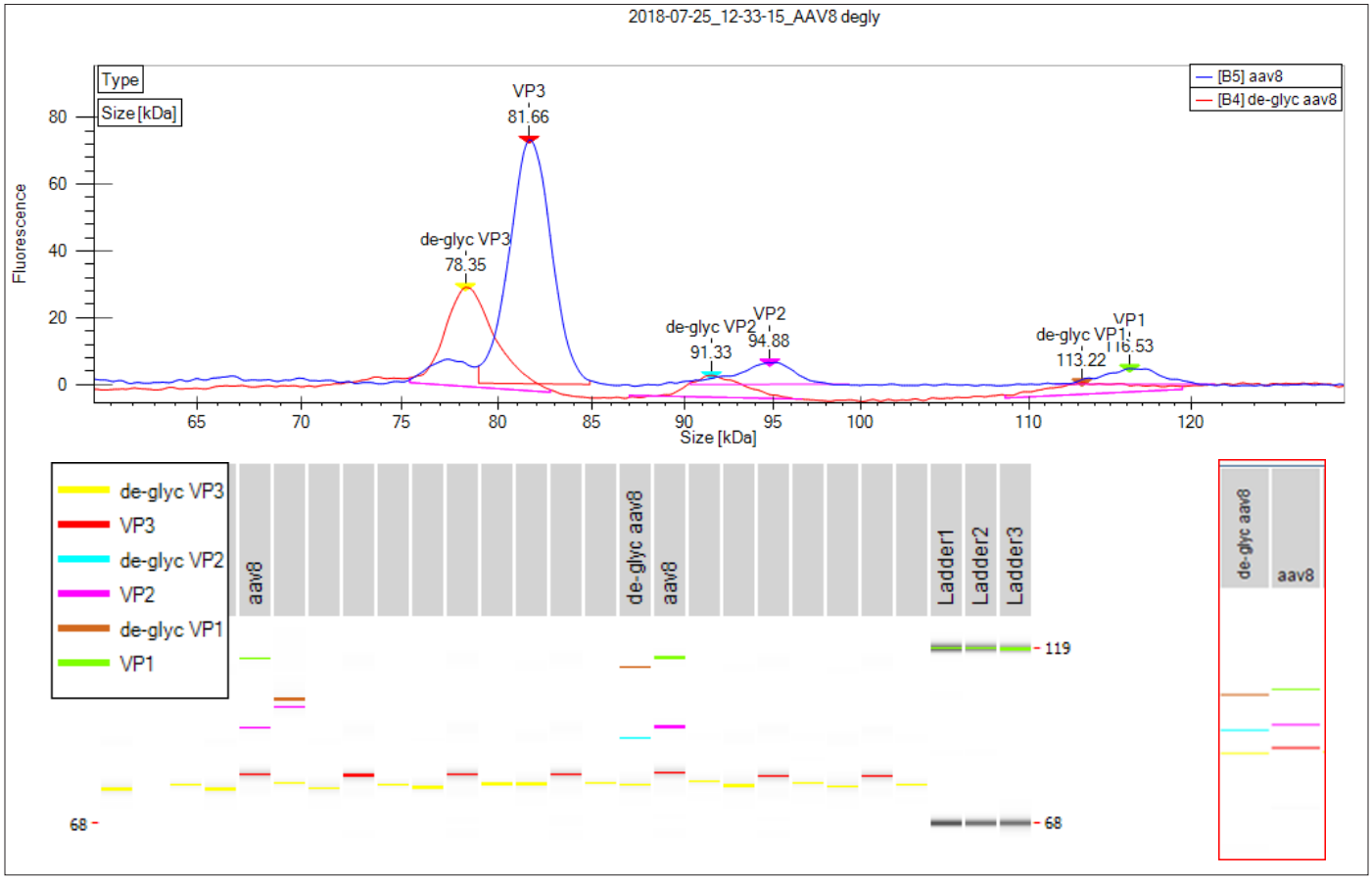


***Figure 4.*** Overlay of broken AAV8 profiles before and after de-glycosylation (experiment 2) along with virtual gel available on PerkinElmer's LabChip® GXII Touch™ protein characterization system.

## Conclusions

In this technical note we presented data on PerkinElmer's ProteinEXact™ assay for the LabChip® GXII Touch™ protein characterization system with AAV samples to 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. Additionally, high-resolution verification of capsid protein glycosylation further benefits production of therapeutically important components.

### 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.


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