APPLIED GENOMICS

APPLICATION NOTE

Rapid Characterization of AAV Capsid Proteins by Microfluidic CE-SDS:

A Quantitative Reproducible Alternative to Gel Electrophoresis

LabChip® GXII Touch™ Protein Characterization System

Introduction

Adeno-associated virus (AAV) particles are used extensively in R&D and clinical applications, such as gene therapy, due to their low immunogenicity in humans. AAV consists of a protein shell which encapsulates a small genome roughly 4.7 kb in size and is dependent on co-infection with helper viruses to replicate. The AAV genome contains *Rep* (replication), *Cap* (capsid), and assembly genes. The *Rep* gene encodes four proteins which are required for genome replication and packaging. The *Cap* gene produces three viral proteins (VP) known as VP1, VP2, and VP3 which form the protective outer shell of the capsid and perform host cell binding.

To visualize VP1, VP2, and VP3, SDS-PAGE with silver stain, a labor and time intensive process, is used to yield qualitative data with poor reproducibility. This procedure can take even the most experienced user hours to complete and many sources of error include: gel overheating, overloading, and inadequate standardization of stain/destaining. A rapid, quantitative, and reproducible alternative utilizes the LabChip® GXII Touch™ HT system[1,2] (Figure 1). This system is a high throughput instrument for the analysis of glycoproteins and nucleic acids via microfluidic capillary electrophoresis in the presence of SDS (CE-SDS). The ProteinExact™ assay for the LabChip® GXII Touch™ system utilizes an on-system calibration step to enable high precision reproducibility along with improved sensitivity and broad sizing range. Sample analysis is completed in 65 seconds.

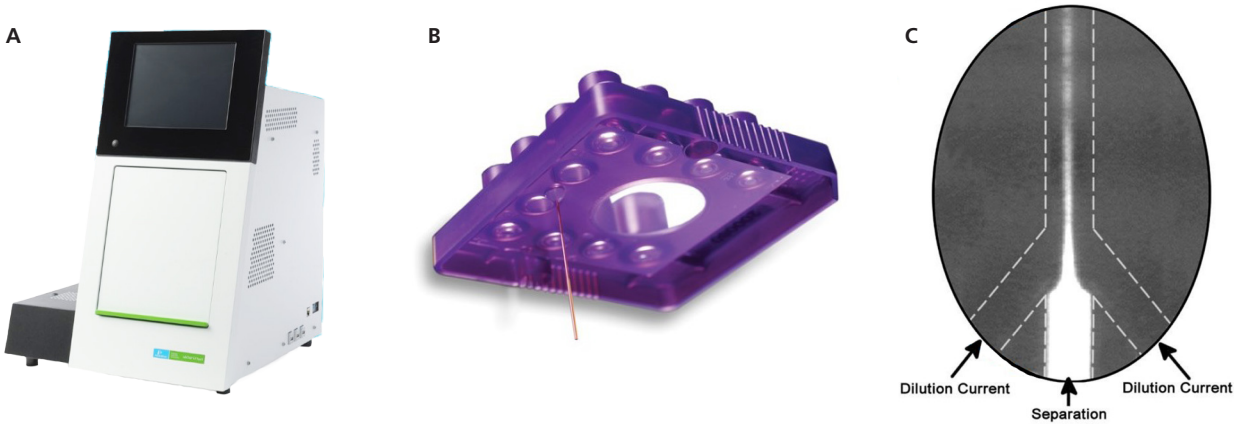

Figure 1: The LabChip® GXII Touch™ instrument (A) uses a quartz sipper chip (B) which allows for testing of up to 400 samples. (C) the fluorescent image shows how proteins are separated and destained in the microfluidic channels.

PerkinElmer®
For the Better

Methods

AAV serotype 8 (AAV8) was purchased from a commercial provider (catalog #A81000, Welgen Inc., Worcester MA). Material was generated in HEK-293 cells, was reported to contain at least 5 x 10^{12} genome copies (GC)/mL, and was supplied in 10% glycerol in PBS.

In accordance with the protocol for the ProteinEXact™ assay[3]:

- In a hardshell 96 well V-bottom SBS plate (catalog #6008870, PerkinElmer, Waltham MA) 2.5 µL of sample was added to 18 µL of nonreducing sample buffer in each well to be tested.
- The plate was sealed, heated at 70°C for 10 minutes, and cooled to room temperature.
- 35 µL of Milli-Q® water (Millipore, Bedford MA), was added to each well and mixed.
- The plate was spun at 1200 RCF at room temperature to remove bubbles and then submitted to analysis on the LabChip® GXII Touch™ HT system.

The thermal stability of AAVs is reported to vary with serotype. The melting temperatures of multiple AAV serotypes have been measured and exhibited a range from 66°C to 90°C[4]. The host cell in which the AAVs were generated and the degree of DNA packing (the empty/full ratio) should also be considered as variables. For these reasons, serotype-specific methods for denaturation of the AAV particles are recommended prior to routine analysis. We suggest that AAV samples be heated in a stepwise manner (2°C steps) from 60°C to 95°C to determine the optimal temperature for complete denaturation without destruction. Heating for 10 minutes has been adequate (data not shown) although we advise that a range from 5 to 15 minutes be explored during method development. Alternatively, the denaturation method that has worked well for SDS-PAGE can be adopted.

Results

The output of the ProteinEXact™ assay run on the LabChip® Reviewer software is a relative electropherogram (egram) with quantitative analysis of size, concentration, and percent contribution of each individual protein relative to the total protein in the sample; those proteins include VP1, VP2, VP3, and substantial protein impurities. The electropherogram along with an in-silico silver stain gel view is provided in Figure 2.

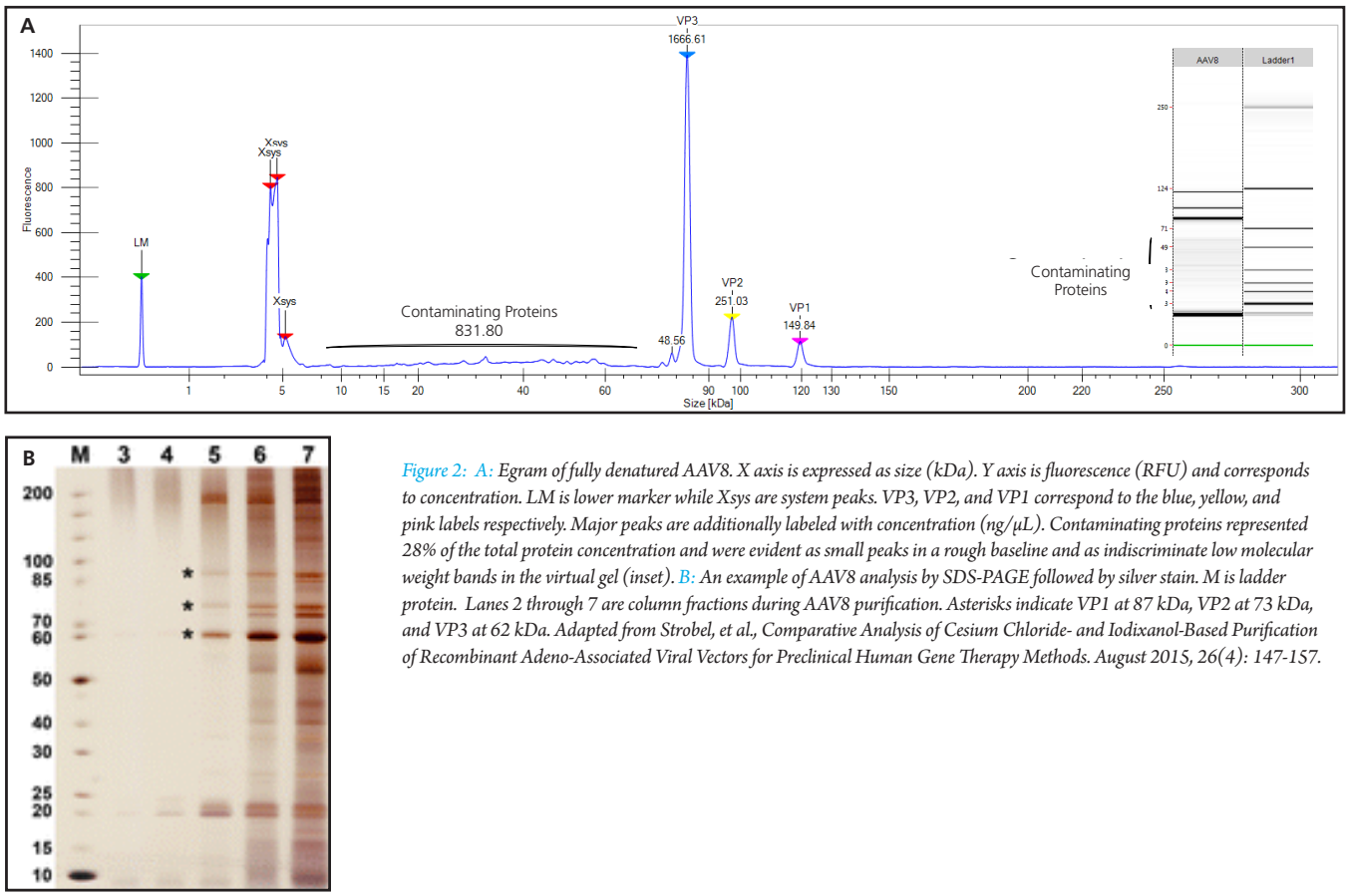

Figure 2: A: Egram of fully denatured AAV8. X axis is expressed as size (kDa). Y axis is fluorescence (RFU) and corresponds to concentration. LM is lower marker while Xsys are system peaks. VP3, VP2, and VP1 correspond to the blue, yellow, and pink labels respectively. Major peaks are additionally labeled with concentration (ng/µL). Contaminating proteins represented 28% of the total protein concentration and were evident as small peaks in a rough baseline and as indiscriminate low molecular weight bands in the virtual gel (inset). B: An example of AAV8 analysis by SDS-PAGE followed by silver stain. M is ladder protein. Lanes 2 through 7 are column fractions during AAV8 purification. Asterisks indicate VP1 at 87 kDa, VP2 at 73 kDa, and VP3 at 62 kDa. Adapted from Strobel, et al., Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Human Gene Therapy Methods. August 2015, 26(4): 147-157.

The corrected area under the curve (AUC) for each protein was automatically calculated by the LabChip® Reviewer software. The AUCs correspond to concentration (ng/µL) and were used to determine the ratio of the VPs within the sample. The reproducibility of the assay (Figure 3) was demonstrated by the overlay of twelve egrams.

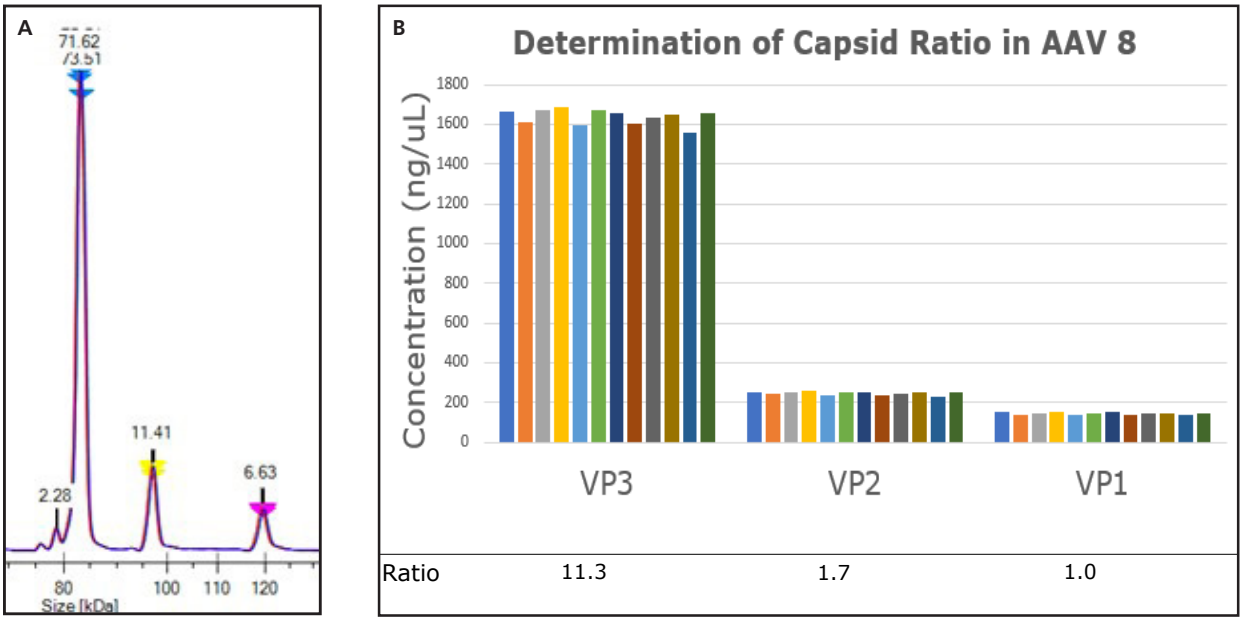

Figure 3: A: Twelve egrams were overlaid to demonstrate reproducibility of the assay of the fully denatured AAV8. X axis is expressed as size (kDa). Y axis is fluorescence (RFU) and corresponds to concentration. VP3, VP2, and VP1 correspond to the blue, yellow, and pink labels respectively. Peaks are labelled with their % contribution to the total protein in the sample. A small peak at 79 kDa represents 2.3% of the total protein. The identity of this fourth peak is not known. B: The calculated ratio of VP3 to VP2 to VP1 was based on the average concentration for each peak, normalized to VP1. The ratio of VP3 to VP2 to VP1 was equal to 11.3 to 1.7 to 1.0.

Discussion

Denaturation of AAV8 and subsequent analysis by microfluidic CE-SDS resulted in a rapid, reproducible, and quantitative measure of VP1, VP2, VP3, along with measurable evidence of contaminating proteins. VP ratios were similar to what was expected (1:1:10). The molecular weights of denatured VP1, VP2, and VP3 were determined to be 119, 97, and 83 kDa respectively as compared to the reported values of 87, 73, and 62 kDa[5]. The apparent MWs between methods can differ, a phenomenon that is routinely observed with monoclonal antibody analysis: mAbs are reported to be 150 kDa by SDS-PAGE but more typically run at 160-170 kDa on any CE-SDS platform. Researchers may want to run each VP individually on both platforms to verify results.

Conclusion

Denaturation of AAV8 and subsequent analysis by microfluidic CE-SDS resulted in a rapid, reproducible, and quantitative measure of VP1, VP2, VP3, along with measurable evidence of contaminating proteins. The ProteinEXact™ assay delivered rapid, reproducible, quantitative results of AAV8 capsid proteins when run on the LabChip® GXII Touch™ HT system. The method is a quantitative alternative to qualitative interpretation of SDS-PAGE. The LabChip® GXII Touch™ HT system aids researchers in studying AAVs, allowing for correlations to be made between *in vitro* and *in vivo* measurements of purity and capsid ratios to *in vivo* observations.

References

1. Smith, MT, *et al.* Establishment and Validation of a Microfluidic Capillary Gel Electrophoresis Platform Method for Purity Analysis of Therapeutic Monoclonal Antibodies. Electrophoresis. 2017 May 38 (9-10):1353-1365.
2. Chunk, WK, *et al.* Effects of Antibody Disulfide Bond Reduction on Purification Process Performance and Final Drug Substance Stability. Biotechnol Bioeng. 2017 Jun;114(6):1264-1274.
3. PerkinElmer. ProteinExact™ HR Assay User Guide, For LabChip® GXII Touch. www.perkinelmer.com/lab-solutions/resources/docs/GDE_ProteinEXact_HR_User_Guide.pdf. Accessed 9-5-2019.
4. Bennett, A. *et al.* Thermal Stability as a Determinant of AAV Serotype Identity. Molecular Therapy: Methods and Clinical Development. 2017, 6:171-182.
5. Backovic, A. *et al*. Capsid Protein Expression and Adeno-Associated Virus Like Particles Assembly in *Saccharomyces cerevisiae.* Microb Cell Fact. 2012; 11: 124.

For more information about the LabChip® GXII Touch™ workstation visit:

perkinelmer-appliedgenomics.com/labchip-gxii

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

To learn more, visit www.perkinelmer-appliedgenomics.com

AG011909_11_AP PKI