APPLICATION NOTE

Considerations in AAV Genome Integrity Analysis: Guidelines from the Use of ssDNA Ladder on LabChip® GX Touch™

LabChip® GX Touch™

AUTHORS

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Introduction

Adeno-associated virus (AAV) is the leading platform of gene delivery for disease treatment due to its long-lasting gene transfection and low immunogenicity. The integrity and purity of the AAV genome, single-stranded DNA (ssDNA) payload in AAV production are critical quality attributes (CQAs) to ensure clinical potency and safety.

A wide array bioanalytical techniques are being used to characterize AAV preparations and evaluate CQAs. Conventional methods such as transmission electron microscopy (TEM) and analytical ultracentrifugation (AUC) offer reliable data on AAV viral particle integrity and aggregation but are limited by throughput and ease of use. Capillary electrophoresis (CE) has also been utilized for viral protein and genome characterization. Higher throughput microfluidic CE platforms such as LabChip® GX Touch™ have enabled miniaturization as well as significantly shorter experimental cycle enhancing productivity and reliability. It incorporates the benefits of miniaturization, integration, and automation for nucleic acid analysis. Here we report the evaluation of a new single-stranded (ssDNA) 7K ladder (1.1 kb to 7.2 kb)[1] as a size standard (PerkinElmer P/N CLS157950) for genomic and other ssDNA fragment analysis on the LabChip® system or other analytical platforms. We share here the performance and analytical sensitivity of the ssDNA 7K ladder as well as a comparative study of the effectiveness of various denaturation methods on double-stranded DNA (dsDNA) using the LabChip® platform. Furthermore, we establish a workflow and recommend best practices for AAV genome integrity analysis.

Main Instruments, Reagents, ssDNA Program & Quick Guide

- LabChip® GXII Touch™ (P/N CLS138160)
- DNA 5K/RNA/CZE HT LabChip (P/N 760435)
- RNA Pico Assay Reagent Kit (P/N CLS960012) or ssDNA 7K Assay Reagent Kit (P/N CLS158169)
- ssDNA 7K Ladder (P/N CLS157950)[1]
- ssDNA 7K Assay Run Program (ssDNA 7K.asyx, P/N CLS158169)[2]
- ssDNA 7K Ladder Quick Guide Using RNA Pico Reagent (P/N CLS157951)[3]
- Zymo Research Oligo Clean & concentrator Kit (spin-column Cat# D4060, 96-well plate Cat# D4023)

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Results

A. Impact of Biochemical Factors on Analytical Performance

I. Buffer diluents affect ssDNA ladder migration

The storage solution (pH=~7.8) of ssDNA 7K ladder contains 50mM K^+, 20mM Tris, 10mM Mg^{2+}, 20mM EDTA, 6% Glycerol. In this section, we investigated peak sharpness and size distribution of ssDNA 7K ladder in different buffer diluents. For this analysis, we prepared samples using 3µL ssDNA 7K ladder stock plus 117µL diluent. As shown in Figure 1, six expected peaks are observed in all diluents, except a high background in formamide. The ladder sample diluted in water shows the best signal intensity, and optimal resolution. It is worth noting that although the overall six-peak distribution patterns are similar in all diluents, buffer diluents have marginal but noticeable effects on peak migration on the LabChip® GX Touch™. For example, ssDNA ladder migrates slower in water as compared to that in TE buffer (pH 8.0). For better size calling and batch-to-batch reliability, we recommend the selection of the same reconstitution buffer or diluent for both the ssDNA ladder and the samples to be analyzed.

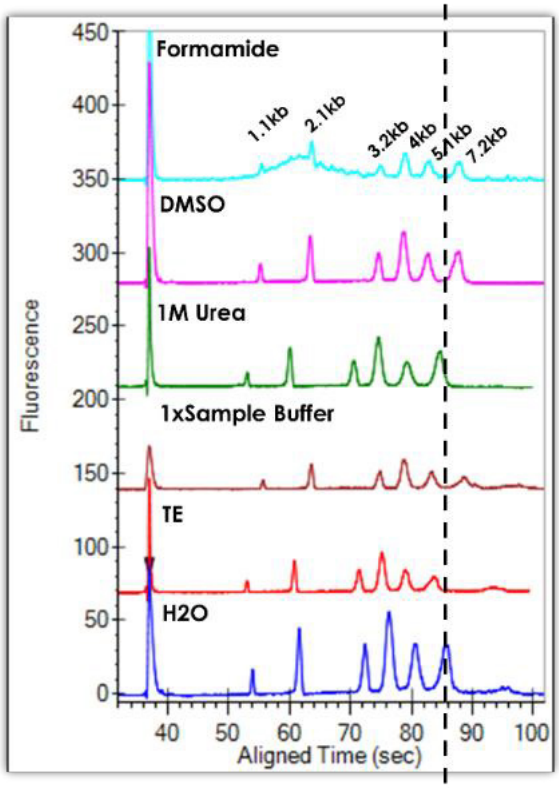

Figure 1. ssDNA 7K ladder peak migration in different diluents. *ssDNA 7K ladder is diluted using water, TE buffer, etc., as labeled in figure. Vertical dashed line is a migration alignment line for easy comparison. Sample Buffer (1x) is provided from RNA Pico Assay Kit (P/N CLS960012). TE buffer: 10mM Tris, 1mM EDTA, pH (8.0).*

II. AAV storage buffer and genome assay sensitivity

AAV samples are usually stored in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) with 10% glycerol, or surfactant (such as 0.01% pluronic F-68). PBS has over 150 mM salt, which is far above recommended sample salt concentration range of any LabChip® nucleic acid analysis products. In this section, we report the investigation of the minimum ssDNA concentration requirement in AAV storage buffer on the LabChip® system. To reduce the risks of microfluidic run failures, only 0.5 x PBS containing 1M Urea solution was conservatively tested. As shown in Figure 2, peak height of 200 nt ssDNA fragment at 4 ng/µL in 0.5 x PBS is < 10 RFU (relative fluorescence unit) and not detectable at 0.4 ng/µL, while at 0.4 ng/µL in TE is still > 20 RFU. These results indicate that the original concentration of PBS compromises the ssDNA (200 nt) detection sensitivity on the LabChip® by around 20-fold, as compared to that observed with TE buffer. These observations point to consideration that AAV samples in PBS for LabChip® workflow represent at least 1.8e12 GC (Genome Copy)/mL (genome size= ~2 kb, ~2 ng/µL) as starting material.

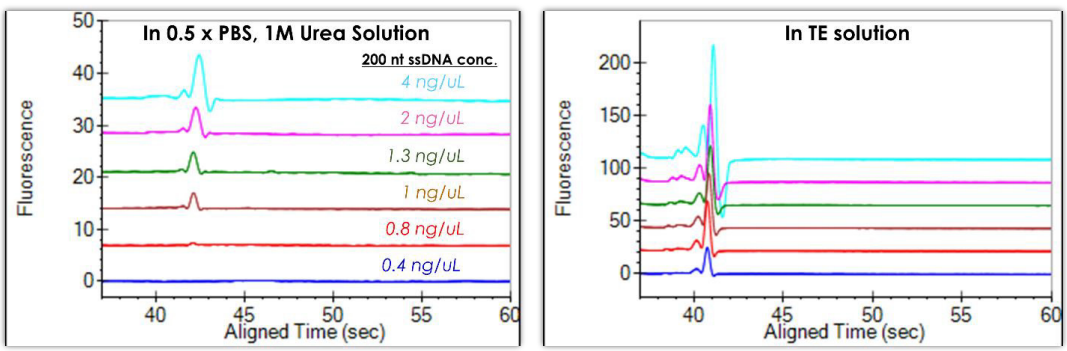

Figure 2. Detection limit of 200 nt ssDNA fragment on LabChip® GX Touch™. *Left panel, ssDNA fragment in 0.5 x PBS 1M Urea solution. Right panel, ssDNA fragment in TE.*

III. Heat efficiently denatures 7.2 kb double-stranded DNA

Denaturing is required for AAV genome sizing analysis for single-stranded fragments. To evaluate the effectiveness of dsDNA denaturing methods, we first prepared one 7.2 kb dsDNA fragment at 100 ng/µL in 50 mM K^+, 20 mM Tris, 10 mM Mg^{2+}, 20 mM EDTA, 5% Glycerol. The dsDNA (7.2 kb) was further diluted at a ratio of 1 to 40 with either water, or TE or 1 M Urea or DMSO (i.e. 3µL dsDNA fragment + 117µL diluent). As shown in Figure 3, without heat treatment and even in the presence of TE buffer or urea, the dsDNA fragment remained undenatured and migrated faster on LabChip® as one peak (around 2 kb) representing the double-stranded form, except the sample treated with DMSO without heat treatment (Figure 3). Upon treatment with heat, although the DMSO-treated sample still shows evidence of dsDNA, the TE-containing and urea-treated samples exhibited a single, distinct band representing complete denaturation. However, the sample diluted with water (containing ~2.5 mM salt) does show a distinct ssDNA peak but with a trailing smear. The smear may be caused by thermal degradation of the DNA. This data also demonstrates the necessity of using ssDNA ladder for ssDNA fragment analysis.

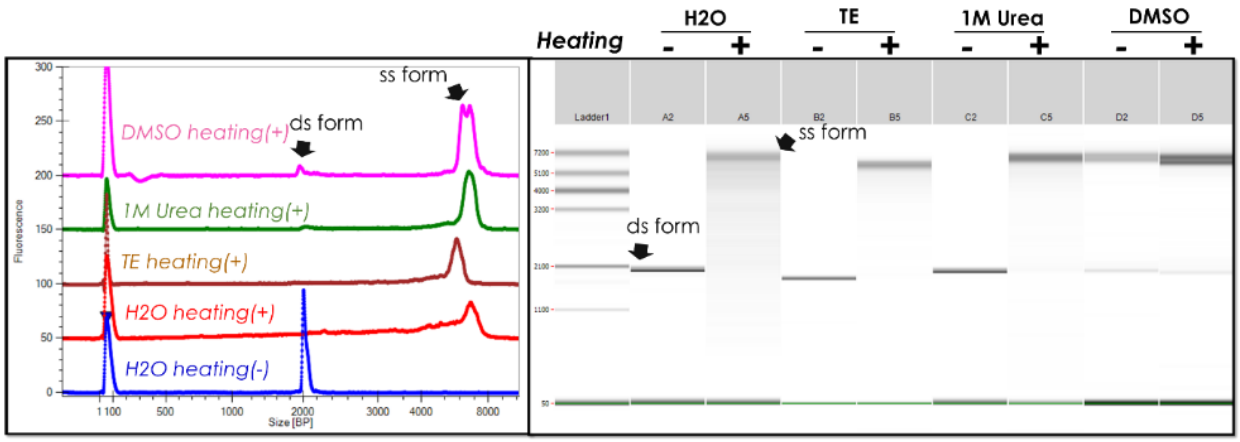

Figure 3. Effectiveness of dsDNA denaturation methods. *Heating (+): treatment at 95°C for 3min, followed by snap cooling on ice for at least 5 min.*

B. AAV Genome Integrity Analysis Workflows

As a result of our observations from the above study, we further explored workflow for AAV genome integrity analysis (Figure 4). AAV samples were first treated with proteinase K (NEB Cat# P8107S, final 0.08 units/µL per reaction) to release the AAV genome from the capsid. Occasionally, to remove any host cell DNA/RNA or plasmid residues on the surface of virus particles, DNase I or Benzonase treatment (optional) can be used prior to proteinase K treatment. We recommend the following nucleic acid purification procedure, which only requires 2-3 min for up to 96 samples. Besides, an advantage to the following purification workflow is a reduction in AAV starting material from ≥ 1.8e12 GC/mL to 3.5e10 GC/mL. Purified samples were eluted using TE (10 mM Tris, 1 mM EDTA, pH=8), heat denatured, and analyzed on the LabChip® GX Touch™ using ssDNA 7K Ladder and the LabChip® RNA Pico reagent.

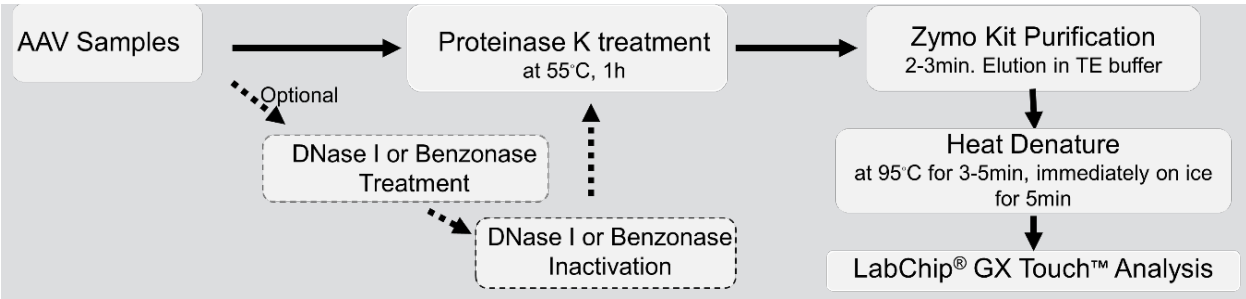

Figure 4. Workflow of AAV Genome Integrity Analysis on GX Touch™. *Steps in dashed boxes are optional.*

To evaluate the workflows in Figure 4, well characterized AAV reference materials (full and empty capsids) from Charles River Laboratories were used as controls. As shown in Figure 5, no peaks were detected in AAV6 empty capsids reference (labeled as CRL-AAV6-RS-ET at 1.79e12 VP/mL), whereas one dominant peak at the expected size position was observed in AAV6 full capsid reference (labeled as CRL-AAV6-RS-FL at 4.1e11 GC/mL). The three samples, AAV2-GOI-1, AAV8-GOI-1 (Lot A), and AAV8-GOI-1 (Lot B) (see footnote* under Figure 5), showed the presence of smeared small fragments (< 500 nt) which are serotype dependent from batch-to-batch. We speculate that these impurities may be nucleic acid contaminants from the host cells. Interestingly, for AAV9-GOI-2 (vendor 2), two prominent peaks were observed, one peak is at ~1.2 kb, which is much smaller compared to intact genome peak at ~2 kb, indicating potential partial capsid in this sample.

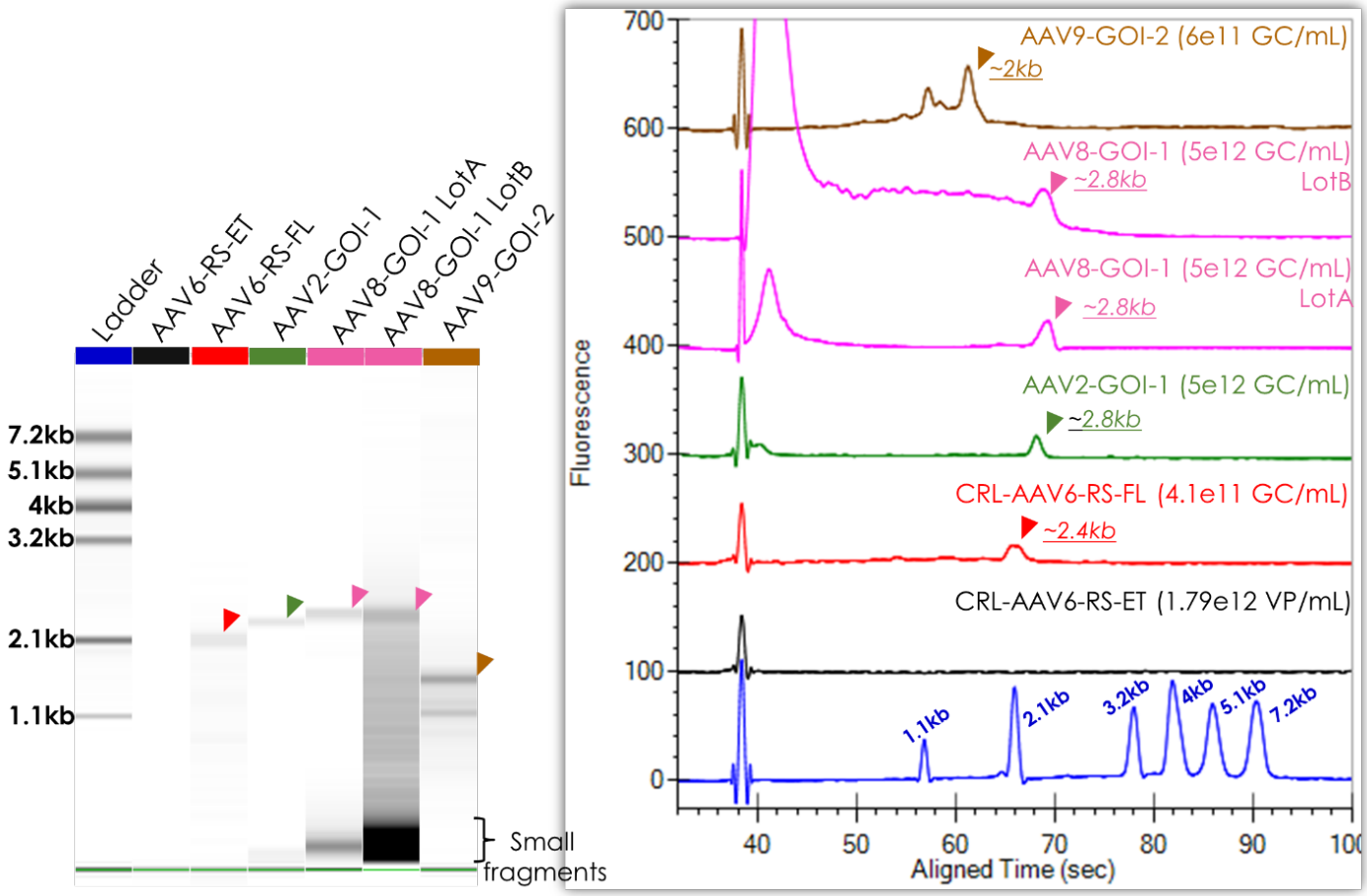

Figure 5: Genome Integrity Analysis of AAV samples using the LabChip® GX Touch™. *Left panel: Gel images. Right panel: Electropherogram. Solid triangles represent expected full-genome peaks. AAV conc. is labeled for each sample. All samples represented here were lysed by proteinase K, purified by Zymo Kit and eluted in 20 µL TE without DNase I or Benzonase treatment. Samples were pipetted into one 96-well plate and sipper height = 4mm were selected for GX Touch™ run. Data shown here is one representative result from triplicates. VP/mL: Viral particles per mL. GOI: Gene-Of-Interest.*

**Three samples (vendor-1) contain same genome (GOI-1: Gene-of-Interest 1), were packaged within different AAV serotypes or manufactured in different batches.*

Summary

Recent advances in bioanalytical technologies and related assay/methods development continue to enhance workflows across novel therapeutic modalities. In addition to the choice of technology and establishment of a streamlined process, delivering on critical quality attributes (CQAs) along the workflows is also dependent upon optimization of experimental factors that can influence data quality, biological relevance, reproducibility, and reliability.

In the above study, we have provided guidance on AAV genome integrity analysis using reference standards such as our recently launched ssDNA ladder in context of various experimental conditions that can further enable success with the LabChip® GX Touch™ platform. We have evaluated parameters such as AAV sample diluents, dsDNA denaturation, buffer salt concentration and enzymatic treatment options to eliminate extraneous DNA/RNA from host cells or plasmids. We recommend addressing above factors to eventually facilitate the development of delivery vectors.

References:

1. *ssDNA 7K ladder kit insert (IFU)*
2. *Software download: https://www.perkinelmer.com/lab-products-and-services/resources/labchip-software-downloads.html.*
3. *ssDNA 7K ladder quick guide using LabChip® RNA Pico reagent*

Technical Contact Information

Please see instrument, reagent and software section listed above for ordering.

Please send questions or requests for user guide and/or evaluation reagent to: **dxsupportamericas@perkinelmer.com** by adding "ssDNA-" at the beginning of the subject line.

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