
Introduction

Single-stranded DNA (ssDNA) is a DNA donor template in adeno-associated virus (AAV)-based gene therapy. For CRISPR Homology-Directed Repair (HDR) mediated gene knock-in, ssDNA has lower rates of random or off-target integration and lower toxicity compared to double-stranded DNA. In addition, ssDNA-based aptamers with high specificity and affinity for their targets provide a new tool for drug delivery and targeted cancer therapy. Rapid growth in the use of ssDNA in biotherapeutics underscores the importance of quality control to ensure the integrity and purity of ssDNA.

ssDNA ladders, as size standards, are a prerequisite for estimating the quality, size, and/or quantity of ssDNA samples. However, for DNA sizes of over 1000 nucleotides, the production of ssDNA ladders is challenging due to technical barriers, lack of similar reference standards, and cost of scale up. Here we present the evaluation and characterization of a novel ssDNA ladder ranging from over 1000 to over 7000 bases using an optimally configured program on PerkinElmer’s microfluidics capillary electrophoresis platform, the LabChip® GX Touch™ Nucleic Acid Analyzer with RNA Pico Reagent. This methodology offers an accurate, higher throughput reference standard and bioanalytical screening solution for ssDNA samples (Figure 1). Since the method is suitable for analyzing up to 7.2kb ssDNAs, it can be used effectively to evaluate critical quality attributes (CQAs) such as relative titer quantification, purity, and integrity of ssDNA from recombinant adeno-associated virus (rAAV) preparations (~4.7kb length) in gene therapy workflows, as well as other molecular applications across new modalities.

Instruments, Reagents and Software

- LabChip GXII Touch instrument (Part# CLS138160)
- DNA 5K/RNA/CZE HT LabChip (Part# 760435)
- RNA Pico Assay Reagent Kit (Part#: CLS960012)
- ssDNA Ladder (80ng/µL, Part# CLS157950)
- ssDNA script (ssDNA-5K.asyx, for evaluation)

Results

ssDNA ladder is more accurate to represent ssDNA charges and molecular sizes

Using the single-strand intercalating agent in RNA Pico reagent, we first investigated migration rate between ssRNA (size standard provided in the kit, CLS960012) and ssDNA (newly developed product, CLS157950) using electrophoresis. As shown in Figure 2, ssDNA is observed to have a much slower migration speed compared to that of ssRNA. For instance, the 6kb ssRNA peak is detected at 56 seconds, while the peak size of ssDNA at a similar time point is only ~ 3.2kb. The results demonstrate that ssRNA ladder is not suitable for size calling of ssDNA fragments, hence, ssDNA ladder should be the ideal choice for ssDNA analysis.

Figure 1: A workflow of ssDNA analysis assay. (A) Chip, Ladder and sample preparation. (B) Program selection on instrument.

Figure 2: Difference of migration time between ssRNA and ssDNA. Gel matrix and RNA ladder are from LabChip RNA Pico Reagent (Part#: CLS960012). HT RNA Pico program is run here on LabChip GX Touch instrument. ssDNA ladder is analyzed as a test sample and run at 2 ng/µL. LM: lower marker. x-axis is time (seconds). Y-axis is fluorescence signal. The size of "1.1K" labeling represents 1100 nucleotides, "K" represents 1000 in this and following figures.
ssDNA ladder shows great purity across platforms

Next, we investigated whether the ssDNA ladder could be used on other electrophoresis platforms. In Figure 3, similar electropherograms representing all 6 peaks were clearly observed on both Agilent’s 2100 Bioanalyzer instrument and the LabChip GX Touch Nucleic Acid Analyzer. However, we noted that neither Agilent’s RNA Pico program nor the LabChip GX Touch RNA Pico microfluidic script is ideal for the 6-peak ssDNA ladder. For instances, the 7.2kb peak is close to the boundary of Agilent’s detection range; while the 4k and 5.1k peaks are partially overlapping on LabChip GX Touch instrument. Further optimization of microfluidic flow control was required, as shown below.

A

B

Figure 3: ssDNA peaks’ consistence on 2100 Bioanalyzer and LabChip GX Touch instrument. (A) 6-peak ssDNA ladder on Agilent 2100 Bioanalyzer using Agilent RNA Pico reagent (cat#5067-1513). (B) 6-peak ssDNA ladder on GX Touch™ using LabChip RNA Pico reagent (Part#: CLS960012). ssDNA ladder running conc. is 2 ng/µL. X-axis is time (seconds). Y-axis is fluorescence signal. Inset plots are simulated Gel image from E-gram.

New run configuration improves ssDNA ladder resolution

To enhance the separation and resolution of the individual ssDNA ladder peaks, we customized the LabChip run program (configuration). All configurations used the same RNA Pico reagents. As shown in Figure 4, configuration3 resulted in the best peak resolution and peak sharpness of ssDNA ladder. Configuration3 underwent additional runs, and the resulting performances are reproducible as shown in Figure 5.

Figure 4: ssDNA ladder peak resolution run by different LabChip run configuration. X-axis is time (seconds). Y-axis is fluorescence signal. LM: lower marker. The plot is the raw E-gram, LabChip Analysis is turned off, peaks are not aligned using lower marker.
Summary

As discussed above, the availability of ssDNA ladder as a reference standard is of significant value for the identification, evaluation, and characterization of ssDNA-based payloads either via viral (e.g., recombinant AAV) or non-viral vehicles (e.g., Lipid nanoparticles, LNPs) in gene therapy or gene editing applications.

We have demonstrated that the newly developed, unique ssDNA ladder (Part# CLS157950) along with the new run configuration on LabChip GX Touch instrument can enable up to 7.2kb ssDNA analysis. Hence, the new size standard will be beneficial for molecular bioanalytical and quality control workflows across gene therapy and other ssDNA related applications.

References

2. Roth et al. 2018 Nature 559, 405-409

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.

Figure 5: Six-peak ssDNA Ladder reproducibility on LabChip GX Touch instrument. X-axis is converted to size (bp). Y-axis is fluorescence signal. Inset plot is simulated Gel image. LM: lower marker.