Application Note

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Analysis of Long ssDNA for Use in CRISPR/Cas HDR with the Agilent 5200 Fragment Analyzer System

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Abstract

Single stranded DNA (ssDNA) templates are becoming increasingly useful in CRISPR/Cas homology directed repair (HDR). Analysis of ssDNA prepared from digestion of dsDNA was compared on the Agilent 5200 Fragment Analyzer system and agarose gel electrophoresis. The high sensitivity and resolution of the 5200 Fragment Analyzer system allowed for detection of small amounts of undigested dsDNA not detectable on the agarose gel.

Introduction

Genetically modifying organisms through CRISPR/Cas has revolutionized the study of genes and their functions. The CRISPR/Cas system can accurately recognize and cleave targeted regions of genomes. This allows for gene knockout through nonhomologous end joining or gene repair/knock-in through homology-directed repair recombination and a repair template. Efficient knock-in of genetic material has traditionally been challenging due to the high prevalence of nonhomologous end joining insertions and or deletions. Recently, single-stranded DNA (ssDNA) templates, ranging from 500 base to kilobase length, have been found to greatly increase the number of homologous recombination events when compared to the traditional double-stranded DNA (dsDNA) templates[1].

The use of strand selective nucleases allows for the creation of long ssDNA from PCR products, permitting researchers to quickly create specific templates. Creation of ssDNA templates requires quality control steps to ensure complete conversion from dsDNA to ssDNA. These quality control steps have traditionally been completed by agarose gel electrophoresis. Use of capillary electrophoresis conserves sample during QC steps and provides more extensive information about the conversion of dsDNA to ssDNA than agarose gel electrophoresis. In this Application Note, we demonstrated the utility of the Agilent 5200 Fragment Analyzer system for use in long ssDNA quality control analysis.

Experimental

The experiments in this study were performed using a 5200 Fragment Analyzer system and can be replicated with comparable results on Agilent 5300 and 5400 Fragment Analyzer systems.

Long ssDNA synthesis

Synthesis of long ssDNA was completed using the Guide-it long ssDNA production system (TaKaRa, #632644) according to manufacturer instructions. Briefly, primers with and without 5' phosphorylation were ordered from Integrated DNA Technologies, Inc. and used to amplify a 1,000 bp product. An aliquot of the PCR product was saved for further analysis on the Agilent 5200 Fragment Analyzer system. The remaining PCR product was purified and subjected to strandase digestion (5 minutes at 37 °C) and deactivated for 5 minutes at 80 °C. In order to model the results of a failed digestion, the PCR product was subjected to reduced strandase digestion (1.5 minutes at 37 °C) and deactivated for 5 minutes at 80 °C.

ssDNA analysis on the 5200 Fragment Analyzer system

dsDNA PCR products were quantified on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) with the dsDNA high sensitivity assay (Thermo Fisher Scientific, #Q32854) and diluted to 0.1 ng/µL. The ssDNA was quantified on the NanoDrop spectrophotometer (Thermo Fisher Scientific) with the ssDNA mode. Analysis of the dsDNA and ssDNA was completed on the 5200 Fragment Analyzer system with the Agilent CRISPR Discovery Gel kit (p/n DNF-910-K1000CP). The ssDNA products were analyzed at around 80 ng/µL.

ssDNA analysis by agarose gel electrophoresis

Approximately 200 ng of dsDNA PCR product and 800 ng of ssDNA was loaded onto a 1 % agarose gel and separated at 100 V for around minutes. TheGeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific, #SM1331) was used (20,000; 10,000; 7,000; 5,000; 4,000; 3,000; 2,000; 1,500; 1,000; 700; 500; 400; 300; 200; 75 bp). After separation, the agarose gel was poststained with ethidium bromide and visualized with a UV transilluminator.

Results and discussion

Accurate sizing of ssDNA on its own is challenging. Therefore, quality control assessment of dsDNA digestion to ssDNA involves size comparison of the starting dsDNA PCR product to the ssDNA end-product. Successful digestion is shown by the dsDNA sizing correctly and the ssDNA sizing smaller. While this can be done on agarose gel electrophoresis, the lack of resolution and sensitivity provides an incomplete picture. The dsDNA PCR starting product and the ssDNA end-product were separated on the 5200 Fragment Analyzer system with the Agilent CRISPR Discovery Gel kit and by agarose gel electrophoresis (Figure 1A and B, respectively). Both methods clearly identified the dsDNA PCR product at the expected size of 1,000 bp and the ssDNA at a smaller size. Analysis of the ssDNA end-product with the 5200 Fragment Analyzer system identified that the majority of the dsDNA sample was converted into ssDNA as indicated by a large peak around 400 bp with a small amount of undigested dsDNA still present at 1,000 bp (Figure 1A; blue trace). Analysis of the ssDNA end-product by agarose gel electrophoresis displayed the appearance of complete digestion to ssDNA with no visible undigested dsDNA present (Figure 1B). Both analysis methods indicated that the dsDNA was digested to ssDNA end-product, suitable for use in homology-directed repair.

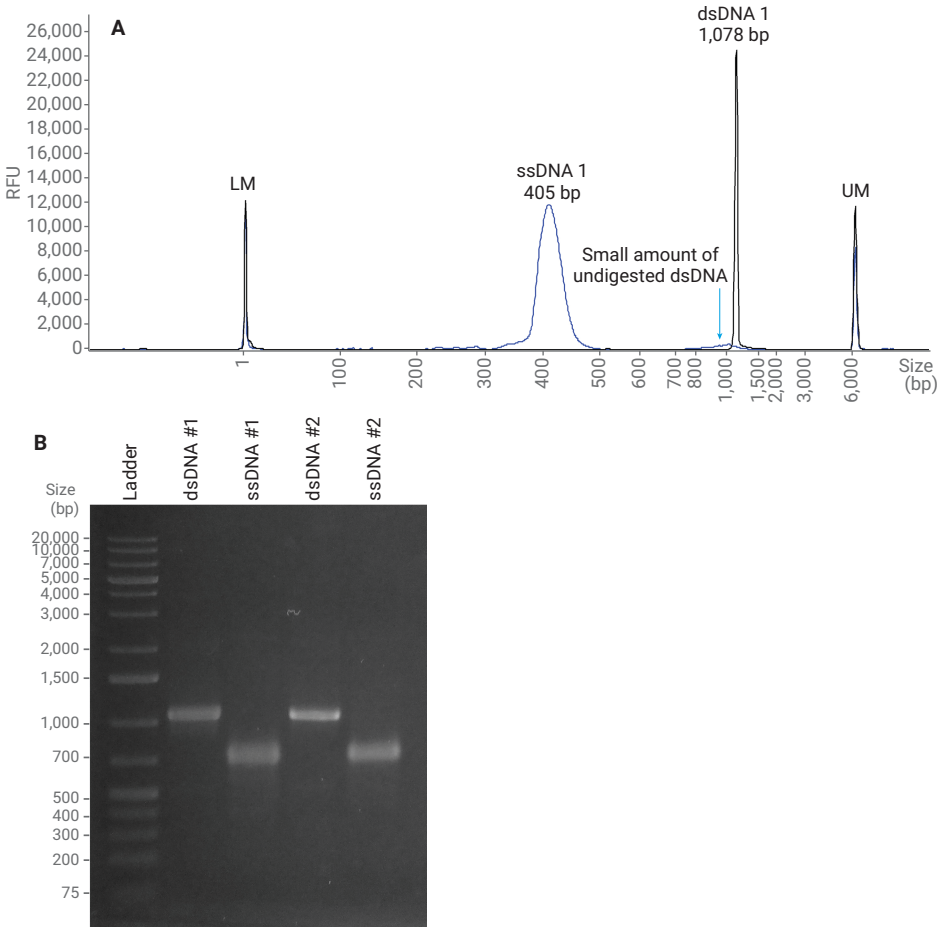

Figure 1. Starting dsDNA PCR fragment and ssDNA end-product after digestion analyzed on the (A) Agilent 5200 Fragment Analyzer system with the Agilent CRISPR Discovery Gel kit, overlay of starting fragment dsDNA 1 (black) and end-product ssDNA 1 (blue). (B) Agarose gel electrophoresis with the GeneRuler 1kb Plus DNA Ladder. LM = lower marker; UM = upper marker.

For demonstration purposes only, dsDNA was partially digested to ssDNA fragments and analyzed on the 5200 Fragment Analyzer system with the CRISPR Discovery Gel kit and on agarose gel electrophoresis. The 5200 Fragment Analyzer system clearly identified undigested dsDNA and ssDNA end-product in both digestions (Figure 2A and B). However, separation of the partially digested dsDNA samples (ssDNA 3 and 4) by agarose gel electrophoresis displayed a smear at a slightly smaller size than the starting dsDNA PCR product (dsDNA 3 and 4) (Figure 2C). Thus, it was hard to determine if the digestion occurred and whether the end-product contained ssDNA, dsDNA, or a combination of dsDNA and ssDNA.

Conclusions

The 5200 Fragment Analyzer system provides rapid, sensitive analysis of the long ssDNA end-product for use in CRISPR/Cas homology directed repair. The greater resolution and sensitivity of the 5200 Fragment Analyzer system compared to agarose gel electrophoresis provides a clearer understanding of sample composition and any small amounts of remaining undigested dsDNA. These aids researchers in deciding if a sample is suitable for downstream applications.

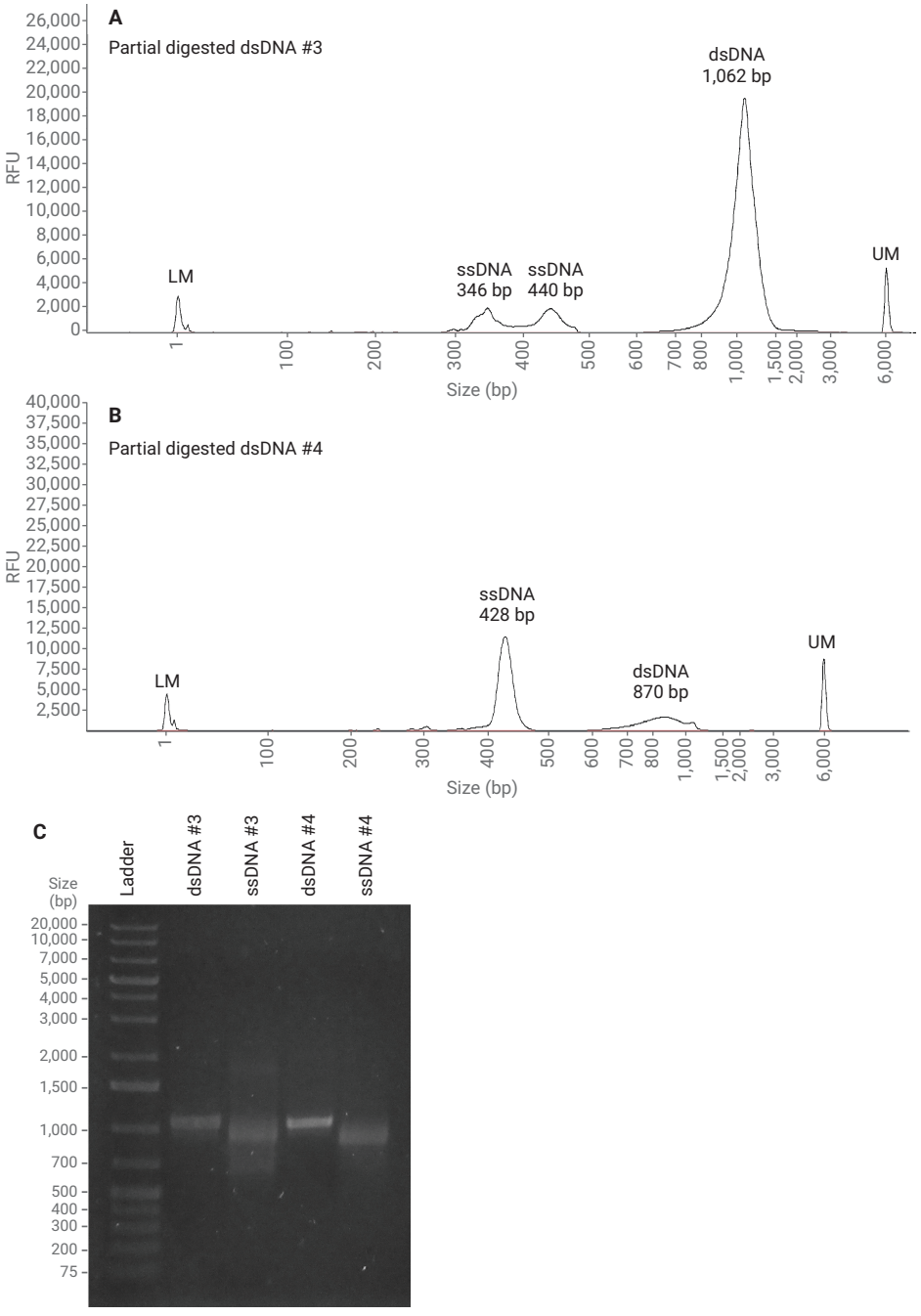

Figure 2. Partial digestion of dsDNA PCR products to ssDNA end-product (A) 3 (B) 4. Both were analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent CRISPR Discovery Gel kit. (C) 3 and 4 on agarose gel electrophoresis. LM = lower marker; UM = upper marker.

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