Application Note

Genomics

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Genomic DNA Handling and the Agilent Femto Pulse System

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Abstract

Genomic DNA (gDNA) integrity is of utmost importance to downstream analysis applications in molecular biology laboratories. Knowing that samples are of high quality helps save time and resources by confirming whether or not the samples are of sufficient quality to generate robust results. Multiple factors can impact gDNA sample integrity, including extraction methods, storage conditions, and mixing techniques. Quality assessment of gDNA is commonly performed by pulsed field gel electrophoresis (PFGE), however, this technique consumes significant quantities of sample and requires lengthy overnight runs. The Agilent Femto Pulse system with the Genomic DNA 165 kb kit offers sizing analysis of high molecular weight (HMW) DNA samples through 165 kb in under 70 minutes, easily replacing PFGE. The high sensitivity and resolution offered by the Femto Pulse allows for even small changes in sizing due to handling to be visualized. This application note explores the impact that different handling protocols can have on gDNA sample integrity and provides recommendations for best practices when performing analysis with the Femto Pulse.

Introduction

Molecular and genetic analyses are foundational to basic, applied, and clinical research, including studies in cancer and inherited diseases, and microbial and infectious diseases. gDNA is commonly used as the input material for numerous workflows including, but not limited to, whole genome and targeted next-generation sequencing (NGS), real-time and targeted PCR, gene editing, and microarray analysis. As gDNA is such an important starting material for vast analysis workflows, ensuring sample quality prior to analysis and at key steps of the protocol is important for generating robust and reliable results.

Performing sample quality control (QC) steps can help a researcher ensure that their samples are of sufficient quality for use in their downstream application and eliminate samples or adjust protocols for samples that may not fit those requirements. Sample quality can be impacted by various factors, including sample storage, multiple freeze-thaw cycles, mixing techniques, and the extraction method[1,2,3]. Degradation can thus occur at many different time points and for many different reasons, including physical shearing from mixing, enzymatic degradation, or improper storage conditions. For example, certain long-read NGS library preparations require gDNA of approximately 20 kb in size. If a sample is significantly larger, it can be sheared to the appropriate size. However, shearing protocols vary based on the size and distribution of the starting gDNA, and it is important to QC the sample following shearing to confirm that it has been sheared to the correct size and has not been over- or undersheared. Sample integrity is important, as degraded samples may impede the downstream workflow and adversely impact data analysis. Thus, guidelines for proper handling of gDNA are essential for the success of downstream applications.

HMW DNA is typically analyzed using PFGE, often requiring lengthy overnight runs and large amounts of sample. The Femto Pulse was designed to address these challenges, enabling automated electrophoresis, run times under 70 minutes, and minimal additional sample handling requirements. The Femto Pulse provides automated pulsed-field capabilities for sizing and integrity analysis of large gDNA through 165 kb with samples easily visualized through digital gel images or electropherograms. Sharp peaks on an electropherogram indicate that a sample is highly intact, with the majority of the sample being fragments of a consistent, similar length. In contrast, a smear appears as a more widely distributed curve on an electropherogram, indicative of fragments of varying lengths compiled together, possibly generated as a result of shearing. Examining the size distribution of a smear can give an indication of a sample's integrity. For example, a sample that has undergone shearing would be expected to show a smear with a smaller average size than the initial sample, as it contains more fragments of a smaller size than the initial gDNA. Protocols for downstream applications using gDNA have varying guidelines for the size, distribution, and integrity of the sample to ensure successful results, making QC of the input sample necessary before proceeding with these sensitive workflows. This application note describes considerations for recommended handling of gDNA samples when using the Femto Pulse, including sample mixing, DNA extraction, storage conditions, and stability.

Experimental

Genomic DNA samples

Commercially available human genomic DNA (gDNA) samples were obtained from Coriell [(p/n NA19238 (Coriell 38) and NA12891 (Coriell 91)] and Promega (p/n G3041). Alternately, gDNA was extracted in-house using three different commercially available kits (A, B, C). Each gDNA sample was diluted to a concentration within the range of the Agilent Genomic DNA 165 kb kit (p/n FP-1002) for analysis with the Agilent Femto Pulse system. Unless otherwise stated, samples were prepared according to the gDNA 165 kb kit manual[4]. Briefly, 2 µL of gDNA is mixed with 18 µL of diluent marker by pipetting up and down two to three times with a wide-bore pipet.

Mixing methods

Different methods of mixing gDNA with diluent marker were investigated. Mixing methods included: pipetting up and down two or three times with a wide-bore pipet (per gDNA 165 kb kit manual[4]), pipetting up and down two or three times with a narrow-bore pipet, vortexing with a touch mixer at various speeds (Vortex 1), and vortexing with two different plate shakers, one set at 3,000 rpm for 2 min (Vortex 2), and the second at 2,400 rpm for 1 min (Vortex 3). Multiple replicates of all gDNA samples were prepared with each mixing type.

Storage temperature

A fresh aliquot of human gDNA (Promega, p/n G3041) was diluted to 300 pg/µL. 100 µL aliquots were prepared and stored at room temperature, 4 °C, and –20 °C for 4 days. The samples were brought to room temperature and analyzed on the Femto Pulse with the gDNA 165 kb kit.

A sample plate was prepared by mixing Promega gDNA with diluent marker and overlaying each well with mineral oil to prevent evaporation. The samples were analyzed on the Femto Pulse with the gDNA 165 kb kit. The sample plate was stored at room temperature for 2 days and analyzed with the gDNA 165 kb kit again to analyze the stability of the sample stored in diluent marker.

Stability study

A Promega gDNA sample was diluted to 490 pg/µL and analyzed on the Femto Pulse with the gDNA 165 kb kit (Time 0). Several 100 µL aliquots were prepared and stored at 4 and –20 °C. Over the following month, the samples were periodically brought to room temperature and mixed two or three times with a wide-bore pipet. Each time, 10 µL of the sample was aliquoted to a new tube and labeled with the number of times the sample had been brought to room temperature, before returning to the storage temperature. After nine such temperature change events, each aliquot was brought to room temperature and prepared according to the gDNA 165 kb kit manual[4]. The samples from days 0 to 9 were analyzed on the Femto Pulse with the gDNA 165 kb kit and the integrity of the samples compared over multiple temperature changes.

Results and discussion

Mixing methods and sample size

Proper handling of gDNA is important to help maintain the integrity of the sample. If a sample is handled roughly, it can lead to degradation. The Femto Pulse is ideal for sizing and quality analysis of large genomic DNAs, with a 70-minute pulsed method that replaces overnight PFGE. With the capability to analyze samples through 165 kb, sample handling is of utmost importance to obtaining reliable sizing data about a sample. According to the Agilent Genomic DNA 165 kb kit manual[4], gDNA should be mixed with diluent marker by gently pipetting up and down with a wide-bore pipet to avoid shearing the sample. To demonstrate the importance of gDNA handling, several samples were mixed in various ways and analyzed on the Femto Pulse with the gDNA 165 kb kit. Mixing methods included pipetting up and down with either a wide or narrow-bore pipet, and vortexing with a touch mixer (Vortex 1) or two different models of plate shakers (Vortex 2 and 3).

Following the gDNA 165 kb kit instructions[4], mixing Promega gDNA with a wide-bore pipet resulted in a sharp peak at about 150 kb. As observed on the Femto Pulse, the resulting peak was slightly smaller when mixed with a narrow-bore pipet or Vortex 2. After mixing with Vortex 1 and 3, sample analysis displayed a smear with a range of about 1,000 to 165,000 bp, and an average size of about 60 kb, indicating that the sample had degraded (Figure 1A). Although Vortex 3 was set at lower rpm for less time than Vortex 2, the smaller size of the sample when mixed in this manner demonstrates the variability that can be seen between different types of vortexes. The large smear seen when the sample was mixed by these methods was not present after mixing with the wide-bore pipet tip, indicating that different methods of mixing resulted in shearing of the Promega gDNA. While mixing gDNA with different methods may be acceptable for other applications, when analyzing samples on the Femto Pulse with the gDNA 165 kb kit, mixing should be performed by wide-bore pipet.

It is possible that the distribution and size of a gDNA sample may impact how easily the DNA strands can be broken. The Promega gDNA sample, which when intact is displayed as a large sized fragment, was observed to exhibit the effects of shearing when mixed with different methods, as indicated by the smaller size fragments and smears seen in Figure 1A.

To determine if the mixing method causes shearing of samples of different sizes, the same techniques were applied to gDNA samples from Coriell (38 and 91), which are known to be smaller than the Promega gDNA. These Coriell samples appear as smears rather than fragments when following the suggested mixing procedures for the gDNA 165 kb kit. Electropherogram overlays generated by the Agilent ProSize data analysis software for Coriell 38 do not show a significant difference in the size of the smear with different mixing methods. However, smear analysis with the ProSize software shows a slight shift in the average size, with the Vortex 1 and 3 mixed samples being smaller (about 35 kb) than those mixed by pipet or Vortex 2 (about 45 kb), suggesting shearing of the sample has occurred (Figure 1B). In contrast, Coriell 91 shows a range of sizes (average smear sizes range from about 30 to 60 kb) and electropherogram patterns with each different mixing method (Figure 1C). Thus, it appears that for the small-sized Coriell gDNA samples, the mixing method does not affect the sample integrity as severely as it does for the larger Promega gDNA.

Together, these data highlight the importance of proper handling of gDNA and indicate that larger-sized gDNA samples may be more impacted by the mixing method than smaller-sized samples as observed by analysis with the Femto Pulse. The mixing method of choice should be gentle enough to maintain the integrity of the sample. Some mixing methods, such as Vortexers 1 and 3, are harsh enough to cause the sample to shear, thereby altering the size of the sample reported by the Femto Pulse when compared to mixing by wide-bore pipet. If the sample prepared in diluent marker is sheared due to the mixing method used, the reported size may not be an accurate reflection of the stock gDNA and may impact any downstream analyses, such as long-read sequencing. Thus, the recommendation for the gDNA 165 kb kit is to mix the samples gently by wide-bore pipet.

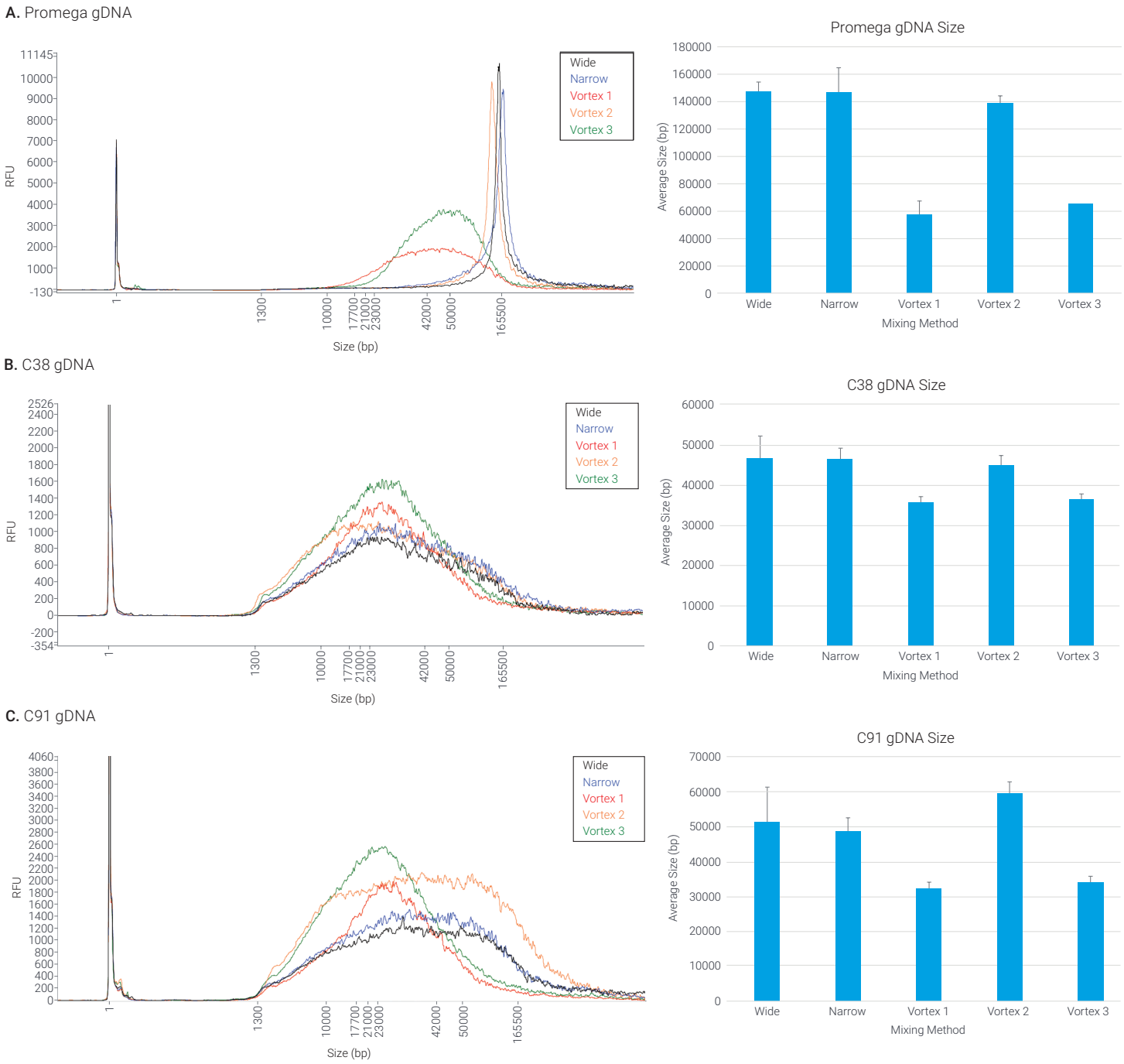

Figure 1. Mixing method impacts the size of gDNA smears. Commercially available gDNA samples ((A) Promega, (B) Coriell 38, and (C) Coriell 91) were mixed with diluent marker using a variety of different mixing techniques, including the recommended wide-bore pipet (black), a narrow-bore pipet (blue), a touch mixer, Vortex 1 (red), and two plate shakers, Vortex 2 (orange), and Vortex 3 (green). Multiple replicates of each sample were analyzed on the Agilent Femto Pulse system with the Genomic DNA 165 kb kit. Representative electropherogram overlays of sample and the average size of each sample with each mixing method are shown.

Mixing methods and extraction kit

It has previously been observed with the Femto Pulse that the gDNA extraction method can impact sample integrity and sizing[2]. To investigate whether the extraction method may be playing a role in the sizing variation with different mixing techniques described above, gDNA from the same sample was extracted using three different commercially available kits (two spin column extraction kits, A and B, and a liquid extraction-based kit, C) and prepared for analysis with the Femto Pulse using the various mixing techniques previously described. The gDNA extracted with kit A resulted in a narrow smear, with a tight distribution covering a small base pair range, and an average size of about 6,000 bp, which was maintained with all mixing techniques (Figure 2A). Extraction kits B and C displayed broader smears, with a wider distribution that covered a larger base pair range and average sizes that varied greatly depending on the mixing technique. gDNA extracted from kit B showed average sample sizes of 18,000 to 30,000 (Figure 2B), while extraction kit C average smear sizes ranged from 13,000 to 19,000 (Figure 2C). With both kits, mixing by wide-bore pipet, narrow-bore pipet, and Vortex 2 resulted in a smear with a large right-side tail. In contrast, mixing by Vortex 1 and 3 displays a more bell-shaped curve, with less area under the curve on the right-hand side, and a larger peak height, indicating that more of the sample is made up of smaller fragments due to shearing of the larger portion of the sample. The large size difference of the gDNA between the extraction kits (Figure 2D) highlights the importance of performing a QC check on input gDNA samples for downstream applications, such as long-read sequencing, which may require protocol adjustments depending on sample size. Additionally, the size variation seen with extraction kits B and C when prepared with different mixing methods provides further evidence that proper gDNA handling is important to determining sample size. It is interesting to note that gDNA from extraction kit A did not have the same reaction to mixing technique as the other two extraction kits. It is possible that this is due to the reagents in the extraction kit or could be due to the already small size of the gDNA offering protection from any further degradation from mixing.

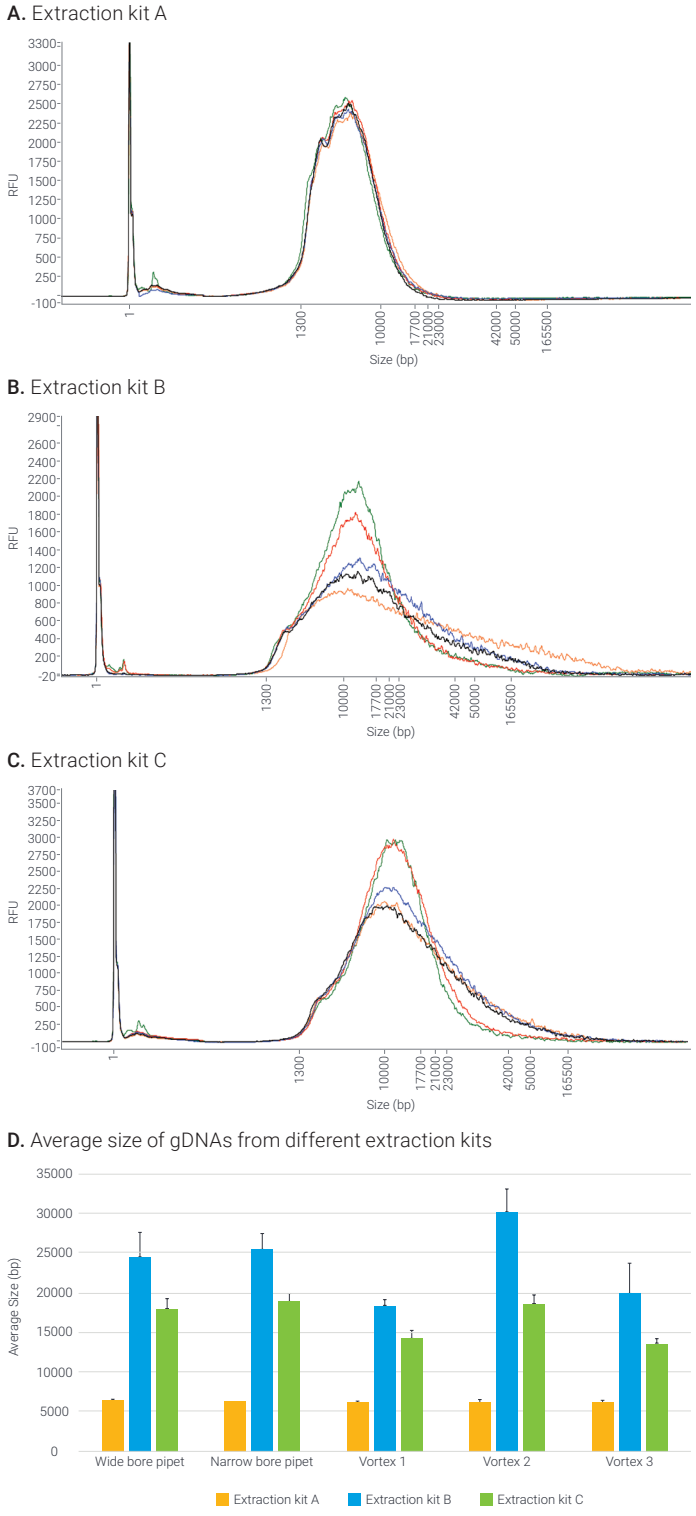

Figure 2. gDNA extraction method may affect sample response to different mixing technique. Aliquots of the same sample were extracted using three different commercially available extraction kits (A, B, C), and were analyzed using the Agilent Femto Pulse system with the Genomic DNA 165 kb kit. The samples were mixed with diluent marker using various different mixing techniques, including the recommended wide-bore pipet (black), a narrow-bore pipet (blue), Vortex 1 (red), Vortex 2 (orange), and Vortex 3 (green). The three extraction kits each resulted in different-sized smears. While the mixing method did not impact the samples from extraction kit A, extraction kits B and C displayed a larger average size when the samples were mixed by wide-bore pipet, narrow-bore pipet, and Vortex 2, and a smaller average size when mixed with Vortex 1 and 3. Representative electropherogram overlays of sample and the average size of each sample (D) over multiple replicates (n=9) with each mixing method are shown.

Short-term sample storage

To investigate the effect that short-term sample storage temperatures have on gDNA, 100 µL aliquots of Promega gDNA at about 490 pg/µL were prepared and stored at room temperature, 4 °C, and –20 °C for four days. Each sample was analyzed in triplicate on the Femto Pulse with the gDNA 165 kb kit. Electropherogram overlays show no difference in the appearance or size of the samples stored at different temperatures, indicating that the gDNA maintains its integrity over short-term storage (Figure 3).

To further investigate gDNA stability, a prepared sample plate was intentionally left at room temperature for two days after the initial run and analyzed again. Promega gDNA samples had been prepared in diluent marker and overlaid with mineral oil to prevent evaporation. Electropherogram overlays of the gDNA showed that while the size of the sample was not affected, the peak height of the gDNA was significantly decreased (n=9) (Figure 4A, B). Additionally, analysis of the ladder wells (n=2) showed that the 165 kb peak was either partially or completely degraded (Figure 4C), making accurate sizing and integrity analysis of the samples difficult. The peak height of the lower marker was not affected, indicating that the reason for the decreased peak heights was not due to run variation. It is for these reasons that the gDNA 165 kb kit manual states that sample plates should be analyzed within one day after preparation to ensure accurate sizing of the sample.

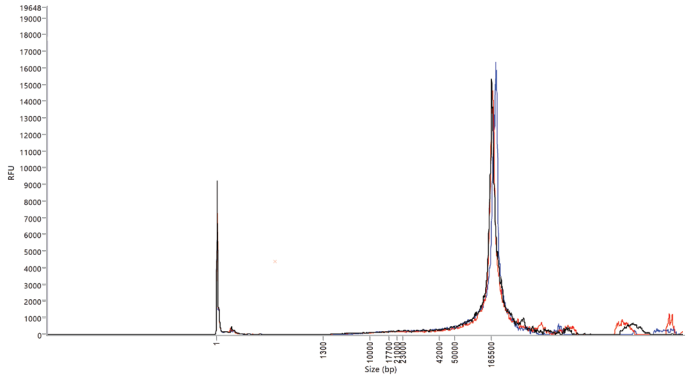

Figure 3. The gDNA integrity is not affected by short-term storage temperature. Promega gDNA was stored for four days at room temperature (RT, black), 4 °C (red), or –20 °C (blue) and analyzed on the Agilent Femto Pulse system with the Genomic DNA 165 kb kit. Electropherogram overlays for all three storage temperatures overlap, with no change in size or distribution, indicating that sample integrity is not affected by short-term storage temperature.

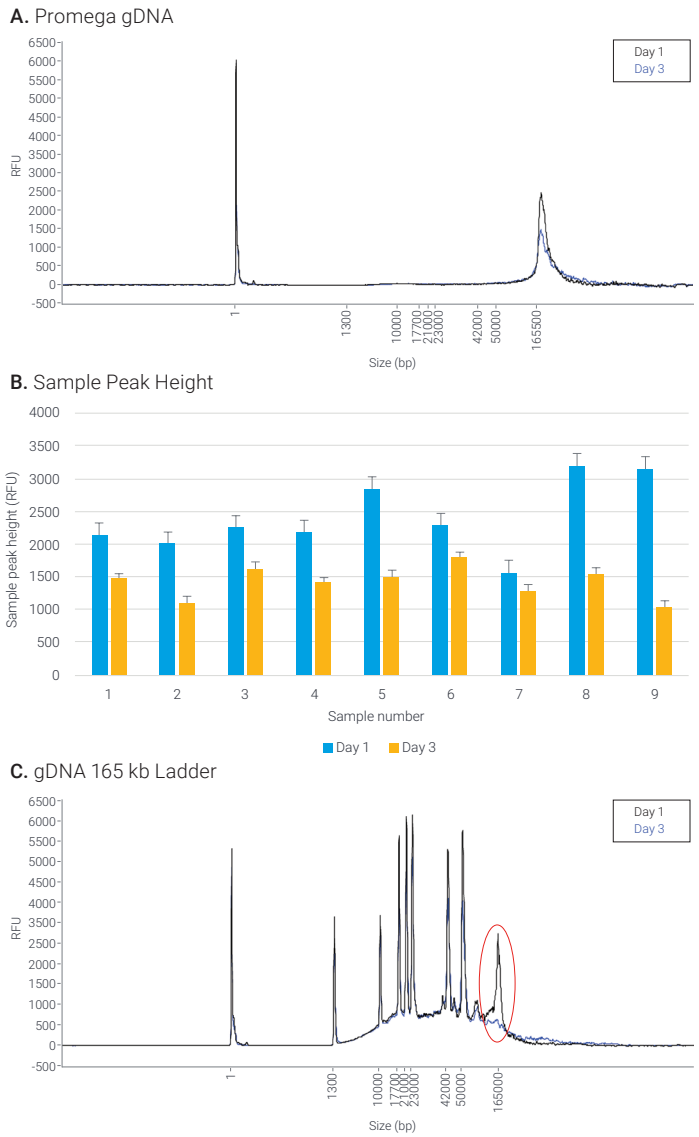

Figure 4. Sample peak heights decrease over time. (A) Promega gDNA samples were analyzed on the Agilent Femto Pulse system with the Genomic DNA 165 kb analysis kit immediately after preparation (black), and after being stored for two days at room temperature (blue). (B) The sample peak heights significantly decreased over multiple replicates (n=9). (C) The gDNA 165 kb Ladder also showed degradation of the 165 kb peak after two days at room temperature (red circle).

gDNA stability

Sample integrity can be affected by multiple temperature changes. To investigate the effect that these events can have on size, 100 µL of Promega gDNA was stored at both 4 °C and –20 °C. Over a period of a month, the samples were periodically brought to room temperature, and a 10 µL aliquot was removed and stored at the same temperature as the initial aliquot. Once the samples had undergone nine such freeze/thaw cycles, each aliquot was prepared for analysis with the Femto Pulse using the gDNA 165 kb kit. The initial sample at Time 0 showed a sharp peak at 165 kb. Over multiple uses with the sample stored at –20 °C, the sample integrity was diverse, with a range of smears ranging from about 1,000 to 165,000 bp (Figure 5A). However, there was no apparent pattern in the degree of degradation over multiple temperature changes. Alternately, when the samples were stored at 4 °C, there was a slight amount of smearing to the left of the main peak, indicating slight degradation of the sample (Figure 5B). However, unlike the samples stored at –20 °C, the sample integrity was maintained throughout multiple temperature change events when the sample was stored at 4 °C and did not degrade further with each use.

The varying patterns seen between the samples stored at 4 and –20 °C for one month indicate that the smearing is likely not due to sample handling at each temperature change, such as pipetting differences, as the same pattern of degradation would have been observed in the 4 °C samples. Thus, it is probable that the amounts of degradation seen in the –20 °C samples were due to the storage temperature. Because gDNA completely freezes at –20 °C, the variation could be because of ice crystals formed in the freezing process, possibly causing physical shearing. Thus, to preserve sample integrity, it is recommended to use freshly extracted gDNA for downstream analysis, or if necessary, to store at 4 °C for short-term storage.

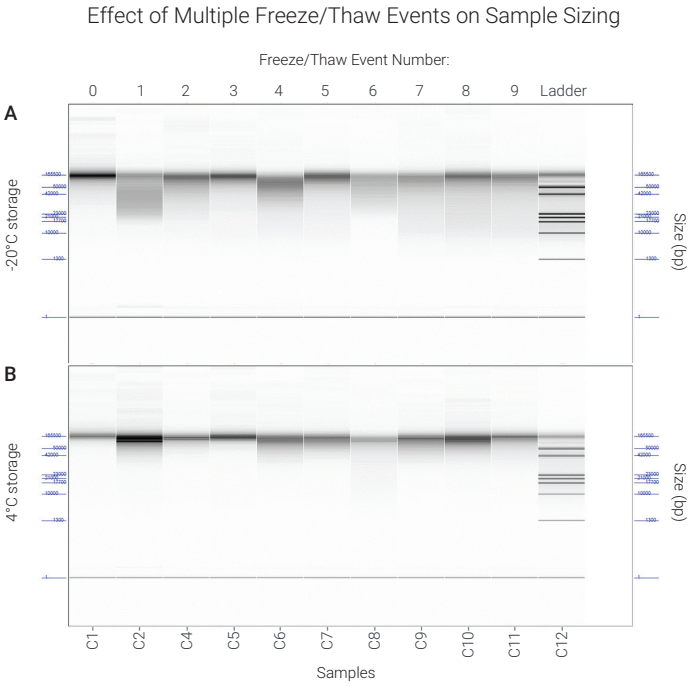

Figure 5. Digital gel images of gDNA samples that have undergone multiple temperature change events, after being taken from storage and brought to room temperature for use. (A) –20 °C sample storage; (B) 4 °C sample storage. The initial sample (0) showed a single peak at 165 kb. Samples stored at –20 °C show a large variation of smearing throughout all freeze/thaw events. At 4 °C, the samples have degraded slightly from day 0, but maintain the same pattern throughout all freeze/thaw events.

Conclusion

Proper handling of genomic DNA (gDNA) should be taken into consideration for the most reliable analysis. Sample handling procedures should be gentle enough to avoid shearing and maintain excellent sample integrity. The Agilent Femto Pulse system offers electrophoretic separations of samples up to 165 kb, with excellent resolution and sizing capabilities that allow for distinction of even the most subtle differences in sample integrity. Guidelines for gDNA handling and analysis on the Femto Pulse include: mixing samples by wide-bore pipet, finding the most appropriate DNA extraction kit for the sample type, using freshly extracted DNA, analyzing sample plates within one day of preparation, and avoiding multiple freeze/thaw cycles. If samples are to be stored, short-term storage at 4 °C is acceptable, and they should be aliquoted to smaller volumes to avoid multiple temperature changes. Following these guidelines will help preserve sample integrity, thereby allowing for the most accurate sample analysis with the Femto Pulse and helping to ensure success in downstream experiments.

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