Application Note

Genomics

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Comparison of Constant- and Pulsed-Field Electrophoresis Technologies for Analysis of High Molecular Weight and Large DNA Fragments

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Abstract

In next-generation sequencing (NGS) workflows, knowing the size and integrity of DNA is important to determine sample input quality and measure the success of library preparation. Electrophoresis is a common technique used in genomic analysis labs to evaluate sample size and quality. Choosing the appropriate electrophoresis technology is critical to achieving successful downstream results. Constant- and pulsed-field electrophoresis technologies are both designed to separate DNA based on size. Constant-field technology is traditionally thought to be best suited for separation of DNA smears that are less than 20 kb, based on previous studies.[1] Beyond 20 kb, DNA smears that are analyzed using constant-field technology may be impacted by the compression of samples, which occurs due to the stacking of DNA within the gel. Pulsed-field technology is capable of separating DNA smears with no known compression effects, as seen in constant-field technology. Agilent offers two capillary electrophoresis instruments that use these different technologies: the Agilent Fragment Analyzer systems use constant-field technology, while the Agilent Femto Pulse system uses both pulsed- and constant-field technologies. The data presented in this application note indicates that while both the Fragment Analyzer and Femto Pulse can be utilized for analysis, the Femto Pulse provides more accurate sizing for high molecular weight (HMW) DNA, genomic DNA (gDNA) and sheared samples, within the range of 10 to 20 kb and above.

Introduction

Quality control (QC) is a crucial component of NGS workflows as it provides valuable information about the size and quality of the input sample, the intermediate steps, and the final library. Knowing the size and quality can help users to make informed decisions on whether their sample is suitable for further downstream processes. QC steps are often performed using electrophoresis to separate the sample based on size. There are many types of electrophoresis technologies, so choosing the most appropriate method for QC is important. QC throughout NGS library preparation can be achieved using both constant-field technologies like the Fragment Analyzer systems, and pulsed-field technologies such as the Femto Pulse system. This application note provides guidance on the appropriate technology to use when analyzing HMW samples and large DNA smears.

Constant-field electrophoresis technology relies on the application of a constant current of electricity running through a gel to separate DNA. This form of electrophoresis is commonly used for smaller DNA fragments and smears less than 20 kb in size.[1] An inherent problem with constant-field electrophoresis is a phenomenon known as compression, which can happen with large DNA samples. Compression results from DNA stacking on top of itself as it moves through the gel gradient. Larger DNA—usually greater than 20 kb in size—is not able to be successfully separated and continues to move as one mass.[1] This lack of separation may cause misrepresentation of the DNA smear size.

Pulsed-field electrophoresis technology is similar to constant-field electrophoresis but applies an oscillating current to the sample during electrophoretic separation. The alternating current of pulsed-field electrophoresis decreases the compression that is seen with constant-field electrophoresis, allowing for more effective separation of larger DNA fragments and smears. The fluctuating current makes pulsed-field technology ideal for QC analysis throughout a long-read sequencing workflow. The lack of compression will cause DNA to separate true to size, providing a clearer picture of a smear's distribution and more accurate sizing of large DNA samples.

The Fragment Analyzer and Femto Pulse systems offer various kits that are suitable for both gDNA analysis and NGS workflow QC. In this application note, the Fragment Analyzer uses the Agilent Genomic DNA 50 kb kit to size samples ranging from 75 to 60,000 bp, while the Femto Pulse employs the Agilent Genomic DNA 165 kb kit and pulsed-field method to size samples between 1.3 and 165 kb. The purpose of this study was to compare the effectiveness of constant- and pulsed-field technology for sizing DNA smear samples within a specific range that both kits share. Specifically, the sizing range tested was between 10 and 20 kb, which is similar to the insert sample range of the PacBio HiFi SMRTbell library, and the shearing range available through the Covaris g-TUBE kit. Initially, the input gDNA was analyzed to confirm sample size and integrity before shearing. Later, QC checks were conducted on the sheared samples to ensure they met the desired size for library preparation. This application note indicates that while the Fragment Analyzer is affected by compression in the 10 to 20 kb size range for smear samples, the Femto Pulse is not. These findings can help researchers choose the most suitable automated electrophoresis instrument for their workflows.

Experimental

Genomic DNA input analysis with constant- and pulsed-field electrophoresis

A commercially available human gDNA sample (Promega, part number G3041) was analyzed to ensure that it met the minimum size requirement of 50 kb for shearing, as recommended by the PacBio HiFi library protocol.[2]
The Agilent 5200 Fragment Analyzer system with the Agilent Genomic DNA 50 kb kit (part number DNF-467-0500) was used for constant-field analysis. The Agilent Femto Pulse system with the Agilent Genomic DNA 165 kb kit (part number FP-1002-0275) was used for pulsed-field analysis.

Sheared DNA sample analysis with constant- and pulsed-field electrophoresis

The gDNA was sheared using a g-TUBE (Covaris, part number 010145) to three different sizes. For each sample, 4 µg of gDNA was added to a g-TUBE for shearing. Samples A and C were prepared following the manufacturer's instructions for shearing to sizes of 10 and 20 kb respectively. Sample B was prepared in the same manner but was centrifuged at 6,000 rpm to achieve a size of approximately 15 kb. Sheared gDNA samples were analyzed on the Fragment Analyzer using the Genomic DNA 50 kb kit and the Agilent HS Large Fragment 50 kb kit (part number DNF-464-0500). The same sheared gDNA samples were run on the Femto Pulse using the Genomic DNA 165 kb kit.

Data Analysis

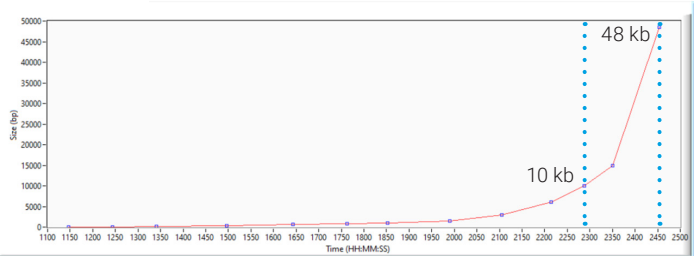
Agilent ProSize data analysis software was used to assess the quality of each sample that was analyzed on the Fragment Analyzer and Femto Pulse. Using smear analysis, each sample was analyzed with a sizing range that incorporated the whole smear.

Results

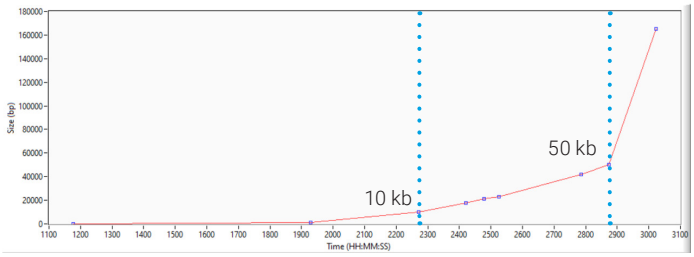
Resolution differences between constant- and pulsed-field separation

To compare constant- and pulsed-field electrophoresis, it is important to consider the separation resolution in the region of interest. To demonstrate the differences in resolution, the slope of the calibration curves for the Fragment Analyzer Genomic DNA 50 kb kit and the Femto Pulse Genomic DNA 165 kb kit ladders were compared (Figure 1). To apply a size to each sample, ProSize software plots time (X-axis) against size (Y-axis) based on the ladder well. Electrophoresis resolution is determined by the length of time required to separate two fragments and the width of the peaks.[3] A longer separation time between two fragments leads to greater resolution and a more gradual slope in the calibration curve of a ladder. A more gradual slope in the calibration curve allows for more accurate sizing because the fragments are more clearly distinguished from one another. To demonstrate this, lines indicating the 10 kb ladder fragments and the 48 or 50 kb ladder fragments for the Fragment Analyzer and Femto Pulse respectively are shown on the size calibration curve in Figure 1. Within this range, the ladder slope for the Fragment Analyzer (Figure 1A) is steeper than that for the Femto Pulse (Figure 1C). The sharp increase in slope for the Fragment Analyzer from 10 to 48 kb demonstrates the lower resolution of constant-field electrophoresis for sizes greater than 10 kb. In contrast, the Femto Pulse has a more gradual slope over a longer period, thus leading to more accurate sizing of samples from 10 to 50 kb.

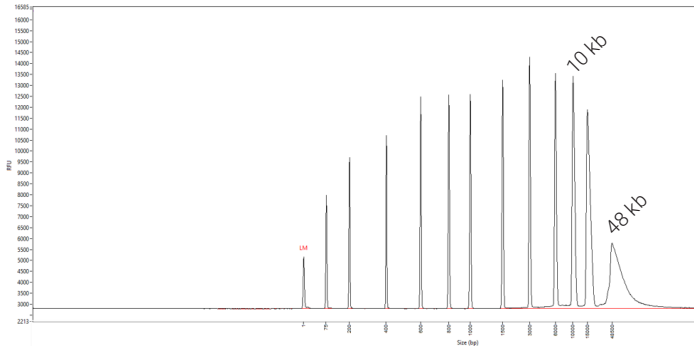
A. Fragment Analyzer Calibration Curve



C. Femto Pulse Calibration Curve



B. Fragment Analyzer Ladder



D. Femto Pulse Ladder

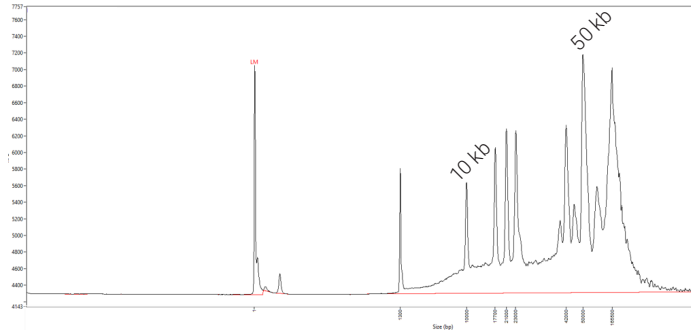


Figure 1. The calibration curves of the ladders for the A) Agilent Fragment Analyzer system and the B) Agilent Femto Pulse system are shown. The blue dotted lines on the curves correspond to the 10 and 48 kb ladder peaks for the C) Agilent Genomic DNA 50 kb kit ladder, analyzed on the Fragment Analyzer, and D) the 10 and 50 kb peaks of the Agilent Genomic DNA 165 kb kit ladder, on the Femto Pulse. The slope of the curves between the dotted lines demonstrate the resolution capabilities of both instruments, with the Femto Pulse having a more gradual slope and thus higher resolution.

Quality control of HMW genomic DNA

QC of the input gDNA is commonly the first step in long-read sequencing library preparation, such as in PacBio's workflow.[4] QC of gDNA can be performed with either constant- or pulsed-field electrophoresis. To display this, the same sample was run on the Fragment Analyzer and the Femto Pulse. The Fragment Analyzer Genomic DNA 50 kb kit is designed to size DNA fragments within a range of 75 bp to 60 kb based on the ladder. When using smear analysis in the ProSize software, sizes larger than the ladder range can be assessed by extrapolation of the ladder calibration curve, enabling some analysis of HMW gDNA samples. The gDNA electropherogram obtained from the Fragment Analyzer is depicted in Figure 2A with a reported smear size of 64 kb.

Pulsed-field technology, which is utilized by the Femto Pulse system using the gDNA 165 kb kit, has a sizing range of 1.3 to 165 kb. Samples larger than the gDNA 165 kb Ladder can be extrapolated beyond the ladder to allow for higher sizing when using the smear analysis function in the ProSize software. Figure 2B displays the electropherogram for the human gDNA analyzed on the Femto Pulse system, with a total smear size of 215 kb.

The smear sizes between the two instruments were measured with a 151 kb difference. Figures 2A and 2B appear visually similar, but the Fragment Analyzer gDNA peak is located close to its largest ladder size on the X-axis at 48 kb, while the Femto Pulse peak is close to its largest ladder size on the X-axis at 165 kb. This shows that the peak locations are distinct from each other when comparing the two technologies because of the difference in scale on the X-axis. Based on the example presented, pulsed-field electrophoresis is the preferred method for analyzing HMW gDNA due to its ability to provide more accurate sizing.

Quality control of sheared genomic DNA

Following assessment of the initial gDNA sample, many NGS protocols recommend shearing the sample to a size appropriate for the sequencing platform. For example, the PacBio HiFi SMRTbell sequencing library preparation protocol requires samples to be sheared to a target size distribution of 15 to 18 kb. In this study, Covaris g-TUBEs were utilized to prepare samples of various sizes. All three samples (A, B, and C) were analyzed on the Fragment Analyzer and Femto Pulse (Figure 3).

The data in Figure 3 demonstrates the comparison in sizing capabilities of the two technologies for all three samples. Sample A, with an expected size of approximately 10 kb, showed little change in average size between constant- and pulsed-field analysis. Sample B, expected to be approximately 15 kb in size, shows a sizing discrepancy between constant- and pulsed-field electrophoresis. The size reported with constant-field analysis for sample B was

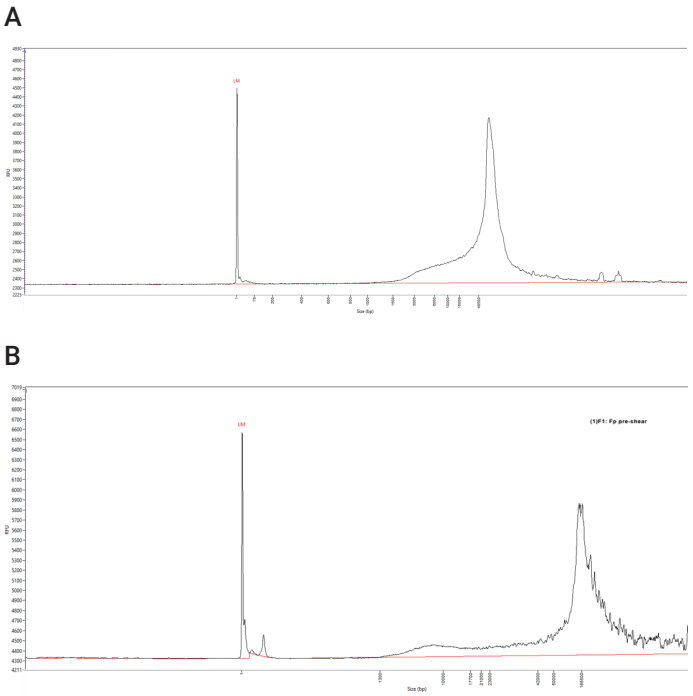
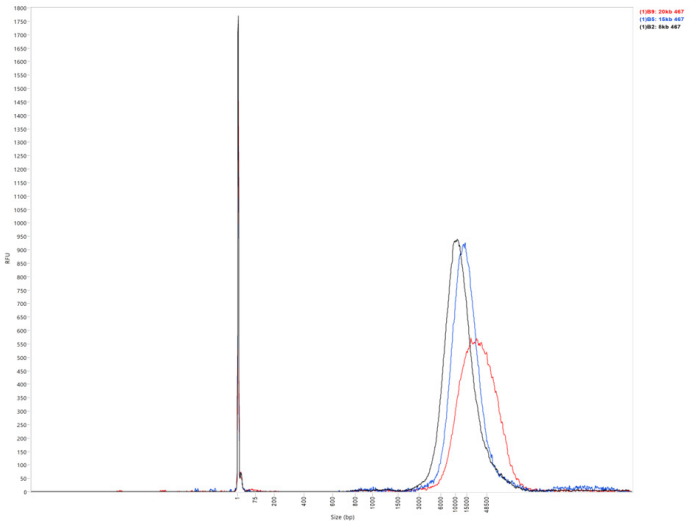


Figure 2. Electropherograms of the same human genomic DNA sample analyzed on the A) Agilent Fragment Analyzer system using the Agilent Genomic DNA 50 kb kit, and B) Agilent Femto Pulse system using the Agilent Genomic DNA 165 kb kit.

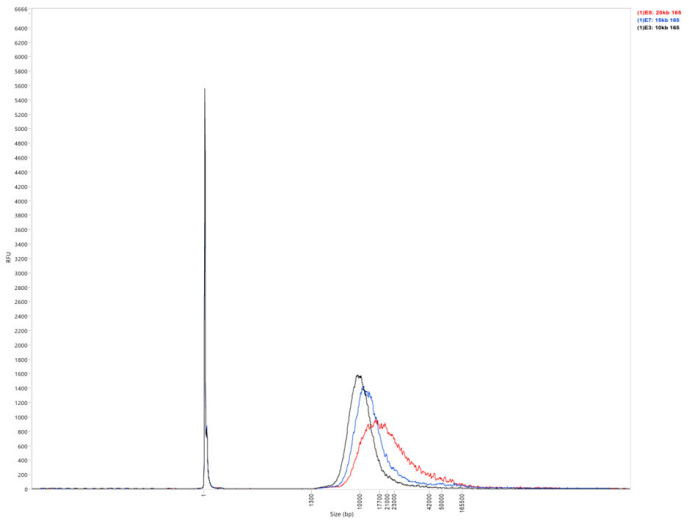
approximately 20 kb. The pulsed-field method sized sample B at approximately 15 kb (Figure 3). The variation between the technologies grows more in sample C, which had an expected size of 20 kb: the constant-field analysis sized the sample at approximately 35 kb, but pulsed-field analysis sized sample C at approximately 21 kb, on average.

The difference in sizing between constant- and pulsed-field technologies becomes apparent with sample B and intensifies as the shear size of sample C is reached. The bar chart in Figure 3C illustrates this difference as sample shear size increases. The observed difference seen is evidence of compression acting on the constant-field sample. Compression is most pronounced in sample C but, presented here, compression begins between approximately 10 and 15 kb smear sizes. Thus, when a sample size is expected to be above 10 kb, it is recommended that pulsed-field electrophoresis technology such as the Femto Pulse is used to achieve the most accurate sizing and integrity analysis of the samples. To further confirm these results, all samples were also run on the Fragment Analyzer with the HS Large Fragment kit. The results shown on the HS Large Fragment kit were consistent with the results shown from the Fragment Analyzer Genomic DNA 50 kb kit (data not shown).

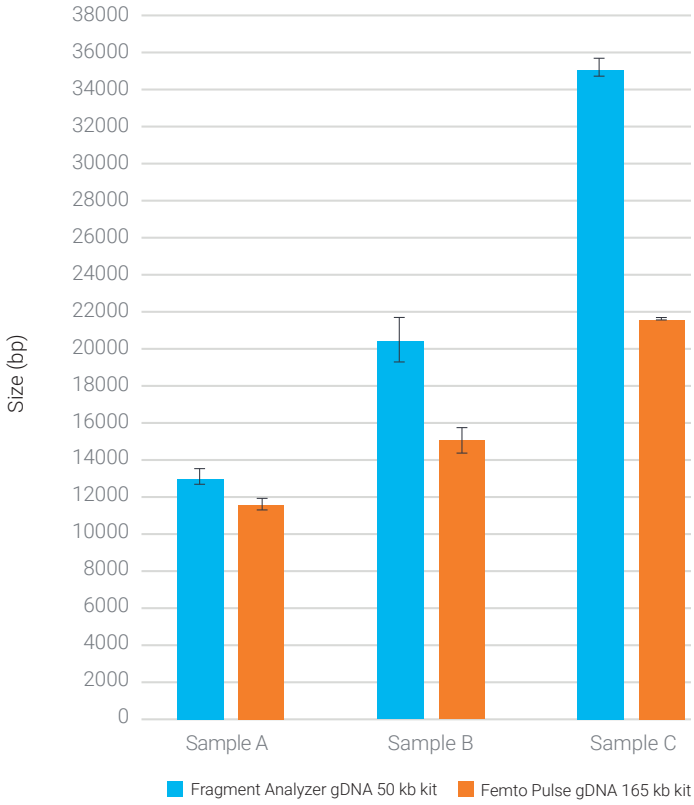
A



B



C



D

	Sample A		Sample B		Sample C	
	Genomic DNA 50 kb kit	Genomic DNA 165 kb kit	Genomic DNA 50 kb kit	Genomic DNA 165 kb kit	Genomic DNA 50 kb kit	Genomic DNA 165 kb kit
Average Size (bp)	13,028	11,649	20,398	15,089	35,142	21,466
%CV	3%	3%	6%	4%	1%	0%

Figure 3. Electropherograms representative of sheared DNA smear analysis with sample A (black), expected size approximately 10 kb; B (blue), expected size approximately 15 kb; and C (red), expected size approximately 20 kb, measured on the A) Agilent Fragment Analyzer system with Agilent Genomic DNA 50 kb kit, and B) Agilent Femto Pulse system with Agilent Genomic DNA 165 kb kit. C) Bar chart comparing the smear size of each sample. D) Table listing the average smear sizes for samples after shearing, measured on the Fragment Analyzer system using the Genomic DNA 50 kb kit, and the Femto Pulse using the Genomic DNA 165 kb kit (*n* = 3).

Conclusion

This application note demonstrates the abilities of constant- and pulsed-field electrophoresis used by Agilent automated electrophoresis systems to size input gDNA and sheared DNA samples in the 10 to 20 kb size range. The results shown here also demonstrate a phenomenon in constant-field electrophoresis called sample compression. Compression causes misrepresentation of the actual size of large DNA smears. The data in this application note showed that as the size of the samples increased, the difference between the reported size of the constant- and pulsed-field data increased. The data also points to compression beginning between the sizes of approximately 10 and 15 kb. Constant-field technology, like the Agilent Fragment Analyzer, provides accurate QC analysis for DNA smears smaller than 10 kb in size, but can still be used for samples up to 60 kb. Pulsed-field technology, like the Agilent Femto Pulse system, is preferred for QC of large DNA greater than 10 kb as it gives better resolution and more accurate size determination.

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PR7001-0900

Published in the USA, June 01, 2023
5994-6103EN

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