

# Best Practices with an Agilent Parallel CE System



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# Introduction

Quality control (QC) assessment is essential to the success of all nucleic acid workflows. Without performing reliable QC steps, the results of downstream applications can be compromised, costing you precious time and resources. The Agilent Fragment Analyzer and Femto Pulse systems, along with the Agilent ProSize data analysis software, provide reliable sizing and quantification of nucleic acid fragments and smears for various applications including PCR, genomic DNA, and NGS library preparations. However, as discussed in this book, certain practices can drastically improve the consistency and accuracy of your results.

This collection of application notes provides the best practices utilized by Agilent for reliable QC analysis and an in depth discussion about the importance of sample quality control during library preparation.

The first application note, Best Quantification Practices with the Agilent 5200 Fragment Analyzer System, compares different mixing techniques as well as the effects of no mixing on sample quantification. For best measurement practices, it is important to choose the correct analysis kit based on the type, size, and concentration of your specific sample, while following the recommended sample mixing practices listed in the kit's manual. Diluting the sample with the correct buffer also ensures consistent, accurate results.

Reliable sizing of nucleic acids is just as essential as quantification during quality control analysis. The second application note, Best Sizing Practices with the Agilent 5200 Fragment Analyzer System, discusses how to obtain the most accurate sizing on the Fragment Analyzer systems. In particular, the differences between peak size and smear

size are examined. ProSize software automatically provides peak size, which is ideal for fragments, while the ProSize Smear Analysis tab provides an average size for smears based on the distribution of the sample over the smear range designated by the user.

The use of independent quality metrics is another important practice that is essential to the success of nucleic acid workflows. Quality metrics remove individual scientists' biases during quality control analysis. Agilent has developed metrics for both RNA and DNA samples. These metrics, along with their uses, are discussed in the final application note, Quality Metrics for Nucleic Acids with the Agilent Fragment Analyzer and Femto Pulse Systems. Evaluating DNA, RNA and genomic DNA quality and integrity is simplified with these quick and user-friendly metrics. Their versatility allows them to be applied to various samples from different organisms, providing reliable assessment of nucleic acid quality and integrity. Following the practices described in this collection will enable researchers to have confidence in the outcomes of their downstream applications.

At the end of the book, Kyle Luttgarm, PhD, discusses the importance of sample QC during library preparation in his interview with *GEN*. Dr. Luttgarm is the product manager for the Agilent parallel CE instruments and has extensive knowledge in this field. During the interview, he addresses a few important topics when it comes to the QC analysis of NGS libraries, that will help ensure successful sequencing results.

We hope you enjoy this book and all the best practices it contains. By following these practices, you can improve your nucleic acid workflow quality control analysis and achieve results you can be proud of.

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# Best Quantification Practices with the Agilent 5200 Fragment Analyzer System

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## Abstract

Quality control checkpoints for concentration are a necessity for many nucleic acid applications and ensures successful outcomes. The Agilent 5200 Fragment Analyzer system provides reliable quantification for fragments and smears with Agilent ProSize data analysis software. This Application Note describes factors that affect quantification and the best practices for achieving accurate quantification of the sample.

## Introduction

Accurate and reliable DNA quantification is essential for many applications such as PCR and library preparations. Reliable results can be obtained when using best measurement practices on the Agilent 5200 Fragment Analyzer system. Best measurement practices can be optimized by choosing the correct analysis kit for the size and concentration of the sample. In addition, thoroughly mixing the sample according to each kit protocol, minimizing pipetting error, and proper sample preparation aids in attaining the best quantification results possible.

## Experimental

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

## Additional Content

Webinar: 'Importance of QC in NGS Library Preparation' hear why EMBL uses the Fragment Analyzer for their QC.

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**Materials**

The following kits were used together with the Agilent 5200 Fragment Analyzer system: Agilent HS NGS Fragment kit (1-6000 bp) (p/n DNF-474), Agilent HS NGS DNA Ladder (p/n DNF-396-U100), Agilent Genomic DNA kit (p/n DNF-487), and the dsDNA HH kit (ThermoFisher Scientific, #Q32854). A Qubit 2.0 fluorometer and a Nanodrop spectrophotometer (both Thermo Fisher Scientific) were used.

**Results and discussion**

**Correct techniques**

Agilent has developed qualitative kits specific for reliable sizing and quality control, and quantitative kits explicitly for accurate quantification, sizing, and quality control. Each kit for the Agilent 5200 Fragment Analyzer system is designed for a specific nucleic acid size and concentration range. It is important to choose the correct kit based on the concentration and size of the sample to achieve the best possible results. Most quantitative kits have a standard sensitivity and high sensitivity version. Generally, the standard sensitivity kits are for higher concentration samples in the nanogram range, while the high sensitivity (HS) kits are for lower concentration samples in the picogram range.

It is important that the sample and ladder preparation protocol is followed for each kit exactly. ProSize data

**Table 1. Recommended mixing protocols for various Agilent 5200 Fragment Analyzer system kits.**

	Swirl while pipetting up and down 10× at 2 µL volume	Swirl while pipetting up and down 10× at 20 µL volume	Plate shaker 3,000 rpm for 2 min	Electronic pipettor 10× at 10 µL volume
Small Fragment kit and HS Small Fragment kit	X	X	X	X
NGS Fragment kit and HS NGS Fragment kit (1-6000 bp)	X	X	X	X
HS Large Fragment 50 kb kit	-	-	X	-
Large Fragment kit	X	X	-	X
HS Large Fragment kit	X	X	X	X

analysis software automatically loads a set of data processing configurations specified for each kit method and are tailored to the sample and ladder preparation protocol described in the corresponding kit manual. ProSize then calculates concentration based on preset volumes of the sample, diluent marker, and ladder for each kit. Any changes in the sample volume, diluent marker volume, or ladder volume will affect the quantification results provided by ProSize.

Bubbles are sometimes introduced to the 96-well plate from preparation and mixing. Bubbles within the sample can interfere with sample uptake into the capillaries. It is advised with all kits to visually check the plate and perform a quick spin to eliminate any possibility of bubbles.

The use of low-bind tubes is recommended when working with nucleic acids. This reduces the loss of

DNA or RNA caused by binding to the side of the tube and ensures more accurate sampling. In addition, use of the recommended 96-well plates ensures that the capillary inlet is set at the proper level in the sample matrix, aiding in reliable sample uptake by the capillaries, since not all plate dimensions are the same.

**Mixing**

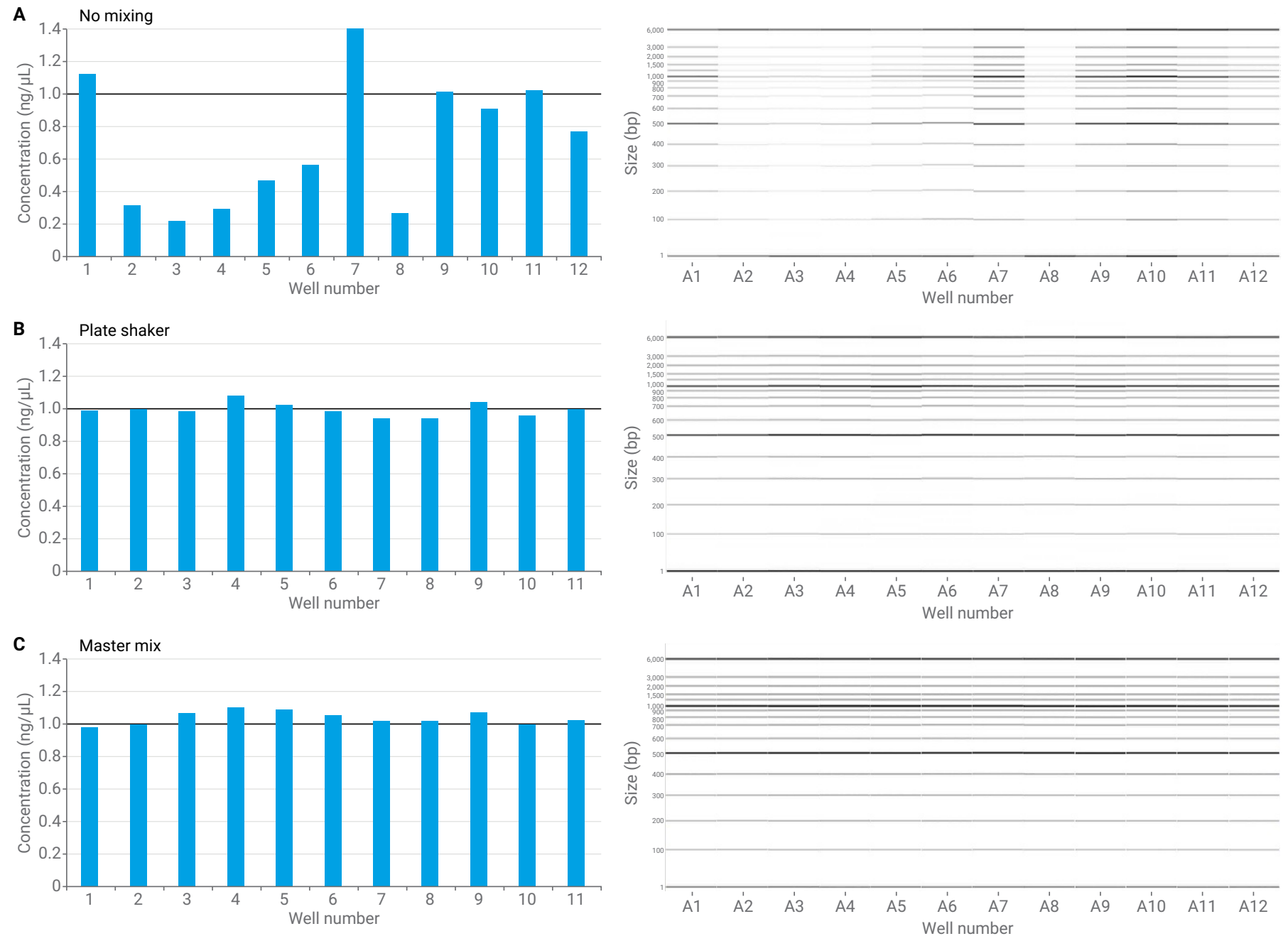
Each kit manual outlines a specific protocol for proper mixing of the sample with the diluent marker (Table 1). All Small Fragment, NGS Fragment, and Large Fragment kit protocols add the diluent marker and then the sample to the 96-well plate followed by mixing.

Mixing allows for homogenous distribution of the sample throughout the well, enabling consistent sample uptake, analysis, and accurate quantification. Sample plates were prepared for analysis with the Agilent HS NGS Fragment kit (1-6000 bp) to

demonstrate the effects mixing has on quantification (Figure 1).

Standard deviation, precision, and accuracy were the parameters for comparing the mixing methods. Standard deviation is used to quantify the amount of variation or dispersion of a set of data values from the mean. Accuracy measures the closeness of a number to the true value and is reported as % error. Precision is the closeness of two or more measurements and is independent of accuracy. A low % CV, representing precision, demonstrates the closeness of the measurements and reliability of the average.

The Agilent HS NGS DNA Ladder was utilized as the sample for the comparison of the mixing techniques. The concentration was determined to be 1.025 ng/μL on the Qubit 2.0 with the dsDNA HS kit. The HS NGS Diluent Marker (1-6000 bp), then sample were individually pipetted into each well of an entire row on a 96-well plate with no additional mixing, followed by separation on the Agilent 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit. After the initial 'no mixing' analysis, the same plate was then vortexed at 3,000 rpm for 2 minutes, followed by analysis on the Agilent 5200 Fragment Analyzer system. Data from samples not mixed showed various concentrations ranging from 0.17 to 0.81 ng/μL resulting in a high standard deviation, low accuracy, and extremely poor precision (high % CV) (Table 2). Mixing the sample plate with the plate shaker improved the homogeneity



**Figure 1. A complete row of Agilent HS NGS DNA Ladder was run on the Agilent 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit (1-6000 bp). Bar graph and gel image. (A) No mixing of the ladder and diluent marker in the plate. (B) Mixing by plate shaker. (C) Master mix of ladder and diluent marker vortexed in a low-bind tube and then pipetted into each well. Mixing is required for reliable quantification of a sample. The known concentration was 1.025 ng/μL.**

of the sample significantly and resulted in a tight concentration range (0.936 to 1.078 ng/μL), a low standard deviation, and an excellent precision (low % CV).

Three more rows were prepared with the same sample and diluent marker. Each row was mixed 10 times according to the suggested mixing protocol listed in the kit manuals: swirling while pipetting up and down at 2 μL volume, swirling while pipetting up and down at 20 μL volume, and electronic pipetting at 10 μL volume (Table 2). All reported accuracies were under 5 % error, with precision values under 6 % CV. Mixing less than 10 times resulted in higher % error and lower precision with the 2 μL volume (data not shown). To avoid variation from mixing techniques, a plate vortexer/shaker is the recommended method for mixing. All recommended mixing methods resulted in quantification precision and accuracy values within the specifications of the Agilent HS NGS Fragment kit.

**Eliminating pipetting errors**

Pipetting introduces intrinsic error into sample handling. It is crucial that pipettes are calibrated, and the proper pipette is used for each volume. For instance, a 2.5 μL pipette would be more accurate for a 1 μL sample than a 20 μL pipette. Appropriate fitting pipette tips also plays a role in eliminating pipetting errors. Proper pipetting techniques ensure excellent precision and consistent quantification. If analyzing replicates, a master mix of the sample and diluent

**Table 2. Lack of mixing greatly effects precision, accuracy, and individual sample quantification as seen with a high standard deviation and % CV. Several mixing methods are suggested in the kit manuals. All mixing methods produce similar precision and accuracy if completed a minimum of 10 times. Samples were separated on the Agilent 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit (1-6000 bp) a: n=6; b: n=12. A master mix provides the most consistent quantification as seen by % error.**

	No mixing <sup>b</sup>	2 μL Pipette mixing 10 <sup>x</sup> <sup>a</sup>	20 μL Pipette mixing 10 <sup>x</sup> <sup>a</sup>	10 μL Electronic pipette mixing 10 <sup>x</sup> <sup>a</sup>	Plate shaker <sup>b</sup>	Master mix vortexed <sup>b</sup>
Average (ng/μL)	0.58	1.07	0.99	0.98	0.99	1.04
Range (ng/μL)	0.17 to 0.81	0.99 to 1.13	0.99 to 1.02	0.92 to 1.04	0.936 to 1.08	0.99 to 1.09
Standard deviation	0.21	0.06	0.02	0.05	0.05	0.036
Precision % CV	36.9 %	5.5 %	2.3 %	5.0 %	4.7 %	3.5 %
Accuracy % error	44 %	4.2 %	3.5 %	4.1 %	3.3 %	1.7 %

marker is recommended to achieve the best quantification precision and accuracy. A master mix involves adding the correct ratio of sample and diluent marker for an entire row to a low-bind tube, followed by vortexing, and aliquoting the mixture into each well. This eliminates error from individually pipetting the sample and diluent marker into each well. As seen in Figure 1C, consistent quantification is achieved across the entire row. The master mix demonstrated baseline reproducibility and provided even better precision and accuracy by eliminating variation from individual pipetting (Table 2).

**Nanodrop versus Qubit**

Quantification on the Agilent 5200 Fragment Analyzer system is often compared to the Qubit fluorometer

or the Nanodrop spectrophotometer. Both the Agilent 5200 Fragment Analyzer system and the Qubit utilize fluorescence detection, while the Nanodrop uses UV-Vis spectrophotometry.

Several factors need to be considered when assessing nucleic acid concentration with UV-Vis spectrophotometry. Nucleic acids such as RNA, ssDNA, and dsDNA all absorb at 260 nm. Contamination sources can absorb at the same wavelengths as nucleic acids. Protein, a common contamination source, can artificially increase DNA concentration through overlap at 260 nm from the 280 nm peak. In addition, proteins with high levels of the amino acids phenylalanine and histidine, or proteins with certain metals bound to them, are more likely to absorb around 260 nm. Furthermore, detergents with



aromatic groups can also absorb in the same region increasing the apparent total concentration of the DNA in the sample.

Fluorescence detection of nucleic acids utilizes a dye that fluoresces only when bound to nucleic acids. Unbound dye does not fluoresce, eliminating unwanted background interference. Fluorescence dyes, in general, have a high specificity for their target molecule. Thus, interference from contaminants such as free proteins and carbohydrates are not an issue when determining nucleic acids concentration. Sheared DNA and human gDNA concentrations were compared on the Nanodrop, Qubit, and Agilent 5200 Fragment Analyzer system (Table 3). Sheared DNA samples (1 to 4) were separated with the Agilent HS NGS Fragment kit (1-6000 bp). The human gDNA samples (5 to 7) were separated with the Agilent Genomic DNA kit. The Nanodrop consistently reported a higher concentration compared to the Qubit and Agilent 5200 Fragment Analyzer system. If comparison of DNA sample concentrations is necessary, we recommend comparing quantification between the Agilent 5200 Fragment Analyzer system and the Qubit due to the use of the same detection method.

**Effects of salt**

Awareness of the salt concentration in samples is important for best quantitative results. The Agilent 5200 Fragment Analyzer system sample preparation protocol recommends that all samples are diluted with 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA) and the chloride salt concentration in the sample must remain below 10 mM. High salt concentrations may cause noisy baselines, sporadic spikes in the electropherogram, and

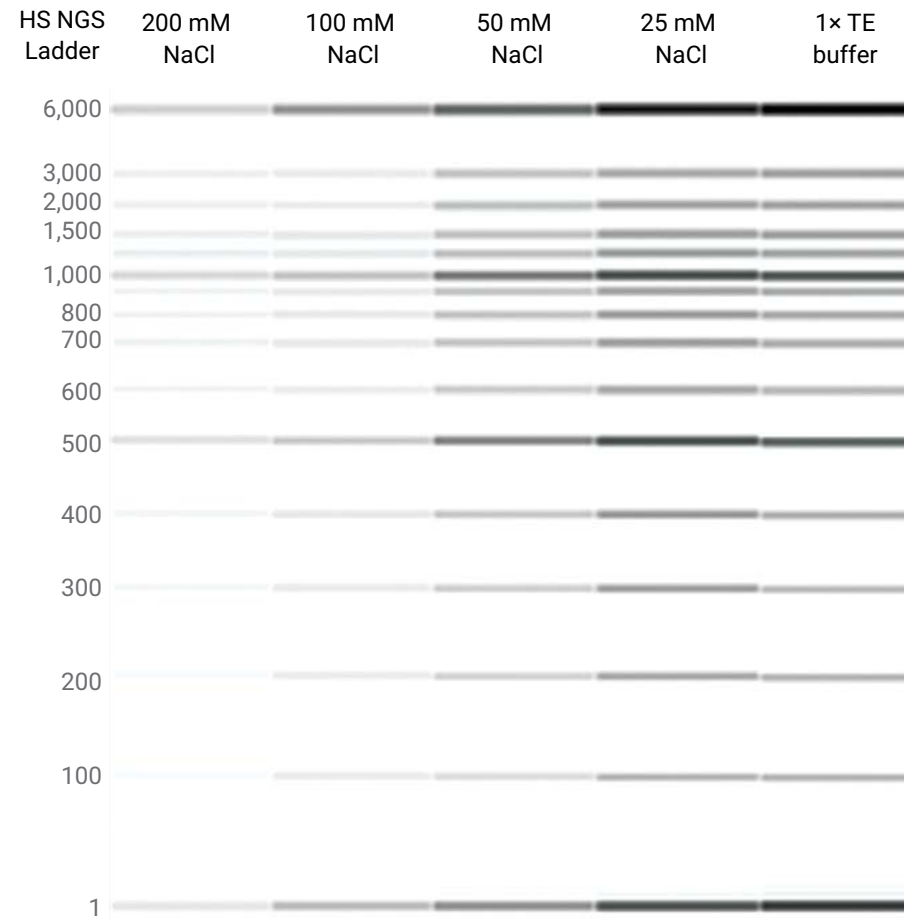
**Table 3. Quantification comparison between the Nanodrop, Qubit, and Agilent 5200 Fragment Analyzer system. The Agilent 5200 Fragment Analyzer system and Qubit both utilize fluorescence for detection. The Qubit is recommended for quantification comparisons with the Agilent 5200 Fragment Analyzer system due to the use of the same detection method. Samples 1 to 4 were sheared DNA separated with the Agilent HS NGS Fragment kit (1-6000 bp) and samples 5 to 7 were human gDNA separated with the Agilent Genomic DNA kit. a: n=2; b: n=3; c: n=18.**

Sample ID	Nanodrop (UV-Vis)		Qubit (fluorescence)		5200 Fragment Analyzer system (fluorescence)	
	Concentration (ng/μL)		Concentration (ng/μL)		Concentration (ng/μL)	
	Avg.	% CV	Avg.	% CV	Avg.	% CV
1	6.15 <sup>a</sup>	3.4 %	2.19 <sup>b</sup>	3.4 %	2.21 <sup>b</sup>	5.9 %
2	5.55 <sup>a</sup>	3.8 %	3.07 <sup>b</sup>	3.3 %	2.37 <sup>b</sup>	1.8 %
3	5.1 <sup>a</sup>	0.0 %	3.30 <sup>b</sup>	1.6 %	3.22 <sup>b</sup>	6.9 %
4	5.2 <sup>a</sup>	5.4 %	3.59 <sup>b</sup>	6.4 %	2.79 <sup>b</sup>	5.8 %
5	197.6 <sup>c</sup>	2.6 %	192 <sup>c</sup>	2.3 %	181.9 <sup>c</sup>	8.1 %
6	100.3 <sup>c</sup>	2.1 %	95.9 <sup>c</sup>	3.4 %	93.1 <sup>c</sup>	7.6 %
7	49.2 <sup>c</sup>	1.7 %	46.5 <sup>c</sup>	4.6 %	43.9 <sup>c</sup>	6.4 %

decreased quantification. The Agilent HS NGS DNA Ladder was mixed with various concentrations of NaCl (200, 100, 50, and 25 mM), separated with the Agilent HS NGS Fragment kit, and demonstrated a proportional decrease in sample concentration with increased levels of salt (Figure 2). Reliable analysis of DNA samples is ensured with the correct salt concentration.

**Input ladder concentration**

ProSize uses the ladder concentration specified in each kit manual to quantify the sample. The ladder for each quantitative kit goes through rigorous quality control checks to certify the concentration with Qubit. However, quantification readings can vary slightly between Qubit instruments due to the surrounding environment, such as the temperature of the lab. ProSize has the flexibility to adjust the concentration of the ladder under the Quantification tab. The ladder concentration can be replaced with the users Qubit concentration measurement in ProSize. Determining the concentration of the Agilent 5200 Fragment Analyzer system ladder and sample with the same Qubit, under the same environmental conditions, will aid in achieving more comparative quantification results between the instruments.



**Figure 2. Agilent HS DNA NGS Ladder analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit (1-6000 bp). Digital gel image of Agilent HS NGS DNA Ladder diluted in 200, 100, 50, and 25 mM NaCl or 1x TE (10 mM Tris-HCl) buffer.**

**Evaporation**

Evaporation of a sample over time is a concern, especially during dry seasons and in drier climates. Evaporation can decrease sample volume, changing the dilution factor and affecting the concentration of a sample. To avoid loss of sample volume, a drop of mineral oil can be added to the top of DNA samples in the 96-well plate. The oil protects the samples from evaporation and retains the ability to prepare and queue multiple runs for unattended analysis.

**Conclusions**

The Agilent 5200 Fragment Analyzer system provides automated analysis of DNA smears, libraries, and fragments. Following the protocols and best mixing practices described in each kit manual and diluting the sample with the correct buffer ensures reliable and accurate DNA quantification. ■

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# Best Sizing Practices with the Agilent 5200 Fragment Analyzer System

## Authors

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## Abstract

Quality control checkpoints for sizing are a necessity for many nucleic acid applications and ensure successful outcomes. The Agilent 5200 Fragment Analyzer system provides reliable sizing for fragments and smears with Agilent ProSize data analysis software. This Application Note describes factors that affect sizing and the best practices for achieving accurate sizing of the sample.

## Introduction

Accurate sizing is essential for quality control analysis of nucleic acids. The Agilent 5200 Fragment Analyzer system offers a wide range of qualitative and quantitative kits that provide reliable sizing for fragments and smears with ProSize data analysis software. Standard deviation, precision, and accuracy were the parameters for evaluating sizing. Standard deviation is used to quantify the amount of variation or dispersion of a set of data values from the mean. Accuracy measures the closeness of a number to the true value and is reported as % error (Equation 1). Precision is the closeness of two or more measurements and is independent of accuracy. A low % CV (coefficient of variance, Equation 2), representing precision, demonstrates the closeness of the measurements and reliability of the average.

$$\% \text{ Error} = \left( \frac{\text{Calculated} - \text{Known}}{\text{Known}} \right) \times 100$$

Equation 1. Accuracy.

$$\% \text{ CV} = \left( \frac{\text{std dev.}}{\text{avg.}} \right) \times 100$$

Equation 2. Precision.

## Additional Content

The Agilent Fragment Analyzer systems let you focus on your nucleic acid QC results while improving your lab efficiency.

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### Experimental

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

Various fragment sizes – 300, 1,000, and 15,000 bp – were analyzed on the 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit (1 to 6000 bp) (p/n DNF-474) and the Agilent HS Large Fragment 50 kb kit (p/n DNF-464) over a concentration range of 3.9 to 500 pg/ $\mu$ L and 4.5 to 600 pg/ $\mu$ L, respectively. A DNA smear was separated with the Agilent HS Small Fragment kit (p/n DNF-477) over a concentration range of 78 to 5,300 pg/ $\mu$ L. The Agilent HS NGS DNA Ladder (p/n DNF-396) was diluted with 200, 100, 50, and 25 mM NaCl or 1 $\times$  TE buffer and separated on the 5200 Fragment Analyzer system with the HS NGS Fragment kit.

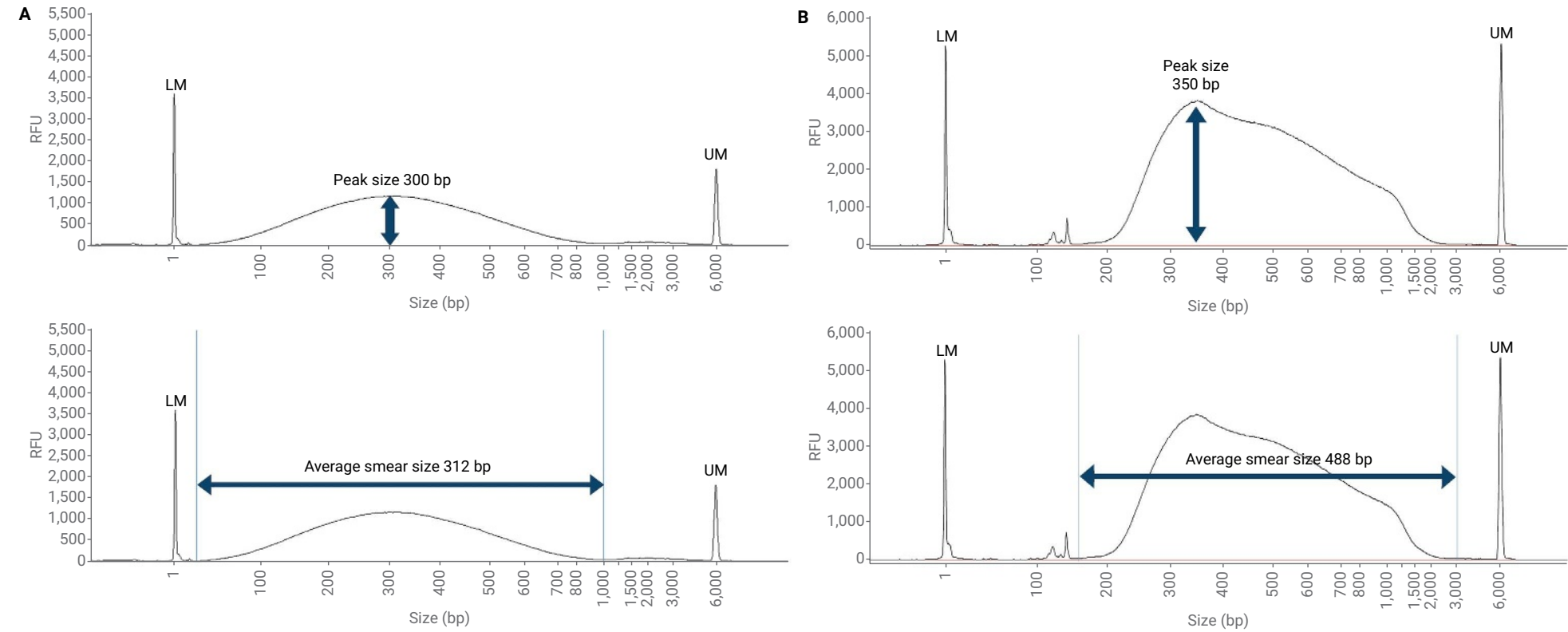
### Results and Discussion

#### Peak size versus smear size

Fragments and smears have different separation profiles on electropherograms. Fragments are displayed as sharp peaks, while smears are broader and lower in height than fragments with the same concentration. DNA libraries vary greatly in size and profile, due to different modes of processing. They often have profiles similar to smears. However, libraries with a small size range can display a profile characteristic of both fragments and smears.

DNA size can be reported as a peak size or smear size. The peak size is the tallest/most concentrated portion of the sample, while the smear size accounts for the entire distribution of the sample over the designated smear range. ProSize automatically reports a peak size for all sample types. The *Smear Analysis* tab in ProSize allows the user to set a base pair range for determination of the average size and concentration of the smear. Peak size is recommended for sizing of DNA fragments.

DNA smears were separated on the 5200 Fragment Analyzer system with the HS NGS Fragment kit in order to compare peak and smear sizing. DNA smears or libraries with a typical bell curve or a uniform distribution on both sides of the highest peak of the sample, reported similar peak and smear sizes (Figure 1A). In contrast, DNA smears with a larger distribution after the highest peak reported a larger smear size compared to the peak size, as expected (Figure 1B). In the same respect, DNA smears with a larger distribution before the highest peak will report a smaller smear size compared to the peak size. Therefore, the most accurate sizing for a smear or library sample can be achieved with the *Smear Analysis* tab on ProSize.



**Figure 1. Peak and smear size of DNA libraries analyzed on the 5200 Fragment Analyzer system with the HS NGS Fragment kit (1 to 6000 bp). (A) A DNA library with a bell-shaped curve will report similar peak and smear sizes. (B) A DNA library with a larger distribution of fragments to the right of the highest peak will have a larger smear size than peak size. Smear analysis is recommended for sizing of all DNA smears and library samples.**

**Sizing under 6,000 bp throughout a dilution series**

The HS NGS Fragment kit has a DNA sizing range of 100 to 6,000 bp. Fragment sizes of 300 and 1,000 bp were analyzed on the 5200 Fragment Analyzer system with the HS NGS Fragment kit throughout the concentration range of the kit (Figure 2A & B). The 300 and 1,000 bp fragments reported consistent sizing of  $299 \pm 1.24$  bp and  $997 \pm 3.35$  bp respectively, with a % error below 0.3 %. Both the 300 and the 1,000 bp fragments had excellent precision values of 0.41 and 0.33 % CV respectively, indicating consistent sizing between the concentration points (Table 1).

	Sizing over concentration range 4.5 to 600 pg/μL	
	300 bp DNF-474	1,000 bp DNF-474
Average (bp)	299 <sup>1</sup>	997 <sup>1</sup>
Range (bp)	297 to 302	992 to 1,001
Standard deviation	1.2	3.3
% CV	0.41 %	0.33 %
% Error	0.06 %	-0.3 %

Table 1. Overview of sizing data for 300 and 1,000 bp fragments over a concentration range separated on the 5200 Fragment Analyzer system with the HS NGS Fragment kit (1 to 6000 bp). 1 n = 144.

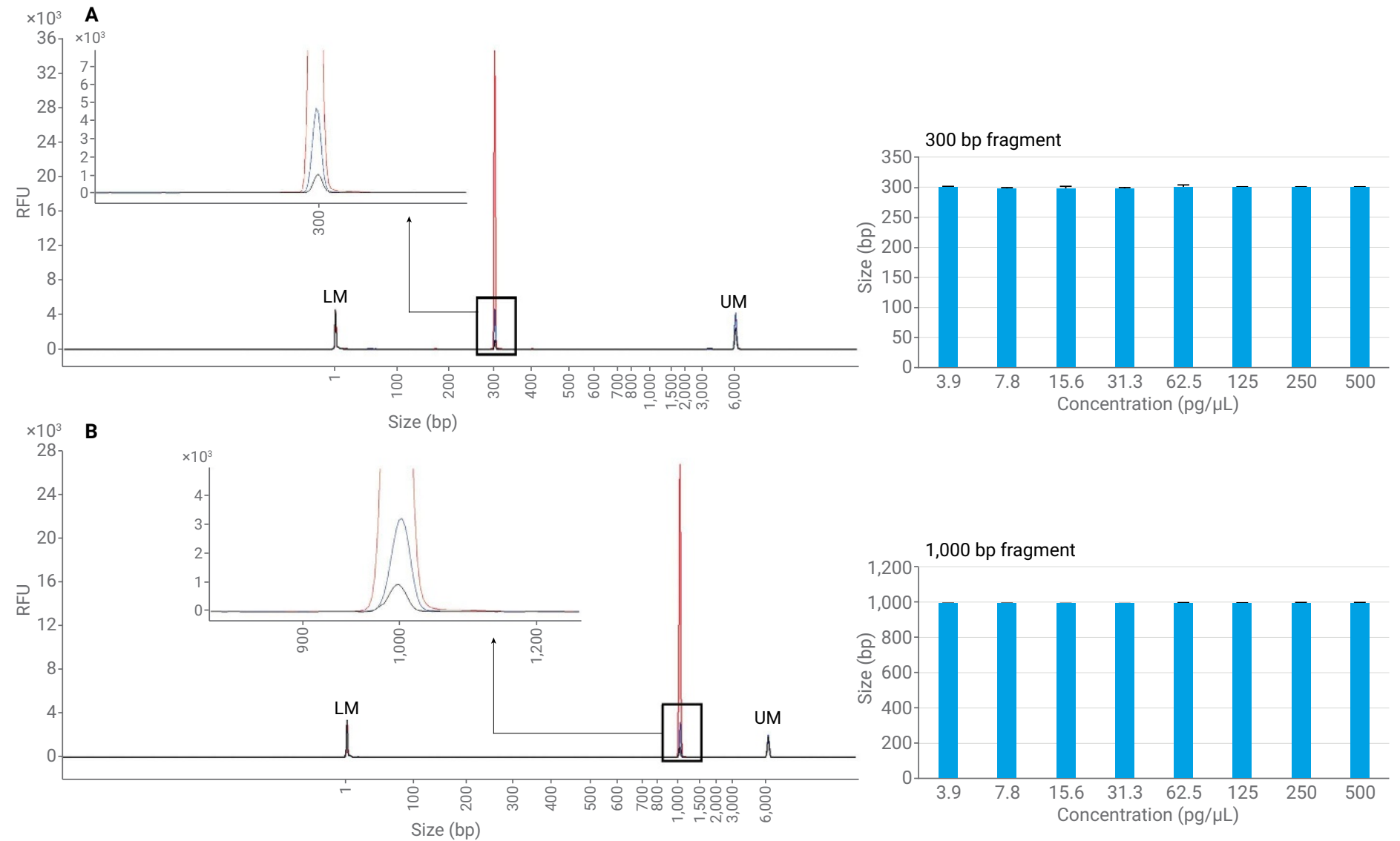
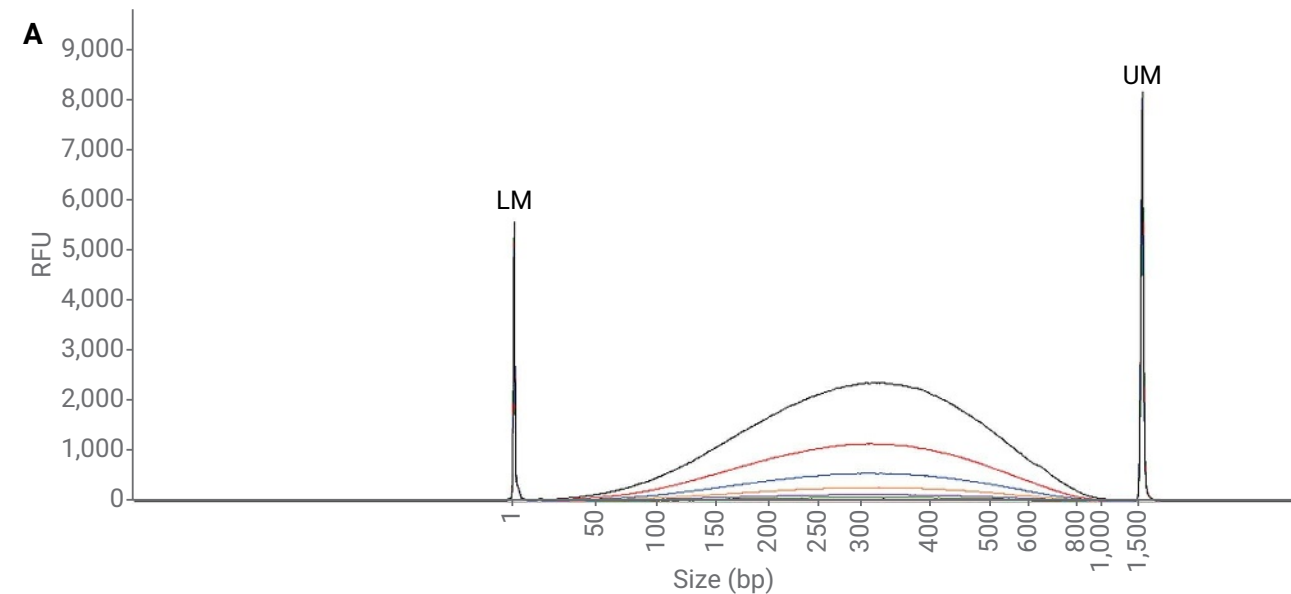


Figure 2. Sizing of 300 and 1,000 bp fragments over a concentration range on the 5200 Fragment Analyzer system with the HS NGS fragment kit (1 to 6,000 bp). A) 300 bp fragment overlay (15.6, 62.5, and 500 pg/μL) and bar graph (3.9 to 500 pg/μL)n = 18. B) 1,000 bp fragment overlay (15.6, 62.5, and 500 pg/μL) and bar graph (3.9 to 500 pg/μL)n = 18. Sizing of fragments under 6,000 bp is consistent, and not affected by concentration on the 5200 Fragment Analyzer system. LM = lower marker; UM = upper marker.

The HS Small Fragment kit has a DNA sizing range of 50 to 1,500 bp. A DNA smear was separated on the 5200 Fragment Analyzer system with the HS Small Fragment kit over a concentration range of 5,300 to 78 pg/μL (Figure 3A). The smear size remained consistent at 295 bp, with no significant change over the entire concentration range of the kit (Figure 3B). Both the Small Fragment kits and NGS Fragment kits provide consistent fragment and smear sizing across the concentration range of the respective kits.



**B**

Smear size over concentration range							
Concentration (pg/μL)	5,300	2,900	1,350	650	320	160	78
Average (bp)	295	294	294	294	296	296	295
Standard deviation	3.5	2.6	3.2	4.3	6.2	5.9	9.4
Precision % CV	1.2 %	0.9 %	1.1 %	1.5 %	2.1 %	2.0 %	3.2 %

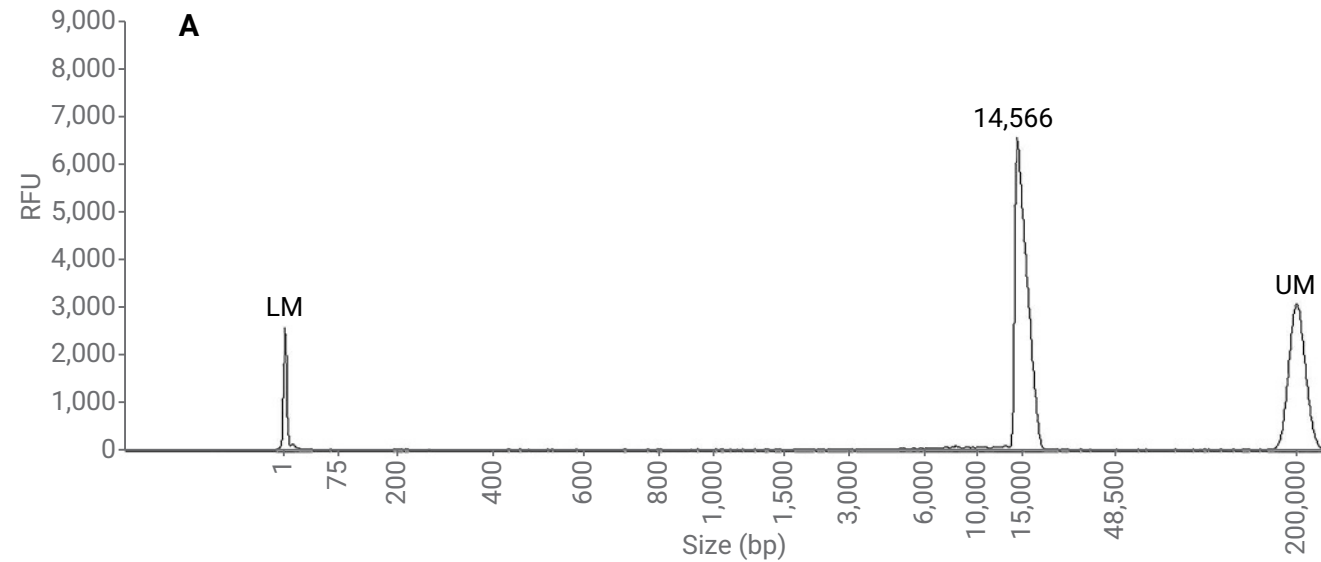
Figure 3. DNA smear separated by the 5200 Fragment Analyzer system with the HS Small Fragment kit over a concentration range of 5,300 to 78 pg/μL. (A) Electropherogram overlay. (B) Table of average smear size, standard deviation, and precision (% CV). DNA smear sizing remained consistent over the entire concentration range. n = 10; LM = lower marker; UM = upper marker.

### Sizing over 6,000 bp

The HS Large Fragment 50 kb kit has a DNA sizing range of 75 to 48,500 bp. To achieve the most reliable and accurate sizing with HS Large Fragment 50 kb kit, an optimal fragment concentration range of 500 to 600 pg/μL and an optimal smear concentration of 1 ng/μL is recommended. A 15,000 bp fragment was separated on the 5200 Fragment Analyzer system with the HS Large Fragment 50 kb kit at the recommended concentration of 600 pg/μL (Figure 4). An average size of 14,566 bp was reported with high accuracy (2.9 % error) and precision (1.4 % CV).

### Effects of salt

Awareness of the salt concentration in samples is important for consistent results. The 5200 Fragment Analyzer system sample preparation protocol recommends that all samples are diluted with 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA) and that the chloride salt concentration in the sample remains less than 10 mM. High salt concentrations may cause noisy baselines, sporadic spikes in the electropherogram, and decreased quantification<sup>1</sup>.



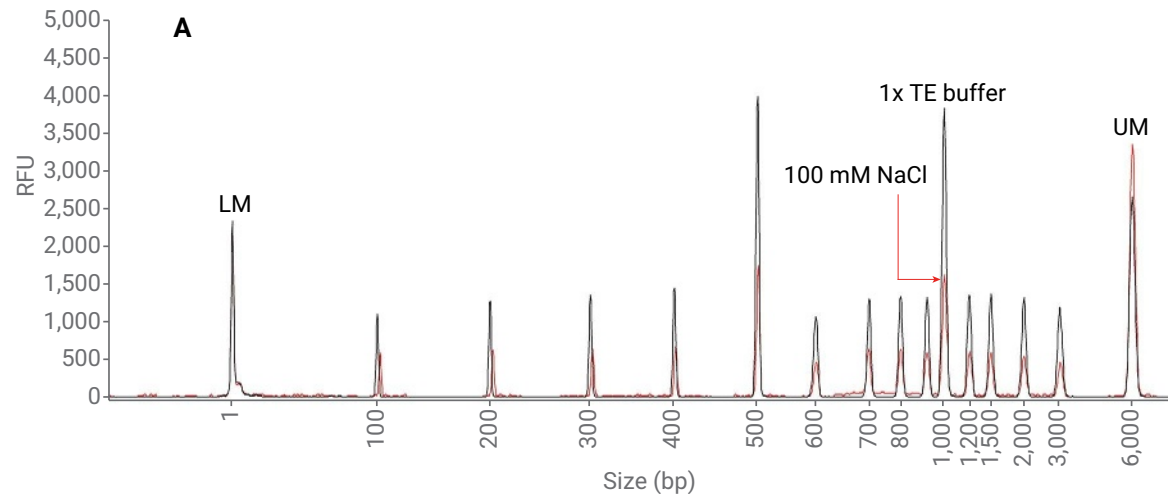
**B**

HS Large Fragment 50kb kit	
15,000 bp at 600 pg/μL	
Average (bp)	14,566
Range (bp)	14,417 to 14,804
Standard deviation	208.0
% CV	1.4 %
% Error	-2.9 %

**Figure 4. Sizing of 15,000 bp fragment at 600 pg/μL on the 5200 Fragment Analyzer system with the HS Large Fragment 50 kb kit. (A) Electropherogram. (B) Table of 15,000 bp fragment sizing data. n = 3; LM = lower marker; UM = upper marker.**



The HS NGS DNA Ladder was diluted in 200, 100, 50, 25 mM NaCl, or 1× TE Buffer (10 mM Tris-HCl) and analyzed on the 5200 Fragment Analyzer system with the HS NGS Ladder kit (Figure 5). Sizing accuracy stayed within 3 % error even at the highest salt concentration. Sizing precision at various salt concentrations remained excellent throughout the sizing range of the HS NGS Fragment kit as noted by the consistently low % CV. Reliable analysis of DNA samples is ensured with the correct salt concentration.



### Conclusions

The 5200 Fragment Analyzer system provides accurate and consistent sizing for DNA fragments and smears. Peak size, ideal for fragments, is automatically provided by ProSize, while the ProSize *Smear Analysis* tab provides an average size for smears based on the distribution of the sample over the designated smear range. Sizing under 6,000 bp is accurate and consistent across the concentration range of the respective kits, with reliable sizing over 6,000 bp occurring with the recommended sample concentrations. High salt concentrations in samples minimally affect sizing, still allowing for exceptional precision and accuracy. ■

### Reference

1. Pocernich, C.; Uthe, J.; Pike, W.; Wong, K-S. Best Quantification Practices with the Agilent 5200 Fragment Analyzer System. *Agilent Technologies Application Note*, publication number 5994-0513EN, 2018.

### B

Sizing of HS NGS DNA Ladder					
Known size (bp)	200 mM NaCl	100 mM NaCl	50 mM NaCl	25 mM NaCl	1× TE Buffer
100	103 ± 0.5	103 ± 0.4	102 ± 0.5	102 ± 0.5	100 ± 0.0
200	204 ± 0.8	204 ± 0.9	203 ± 0.4	203 ± 0.5	201 ± 0.5
300	304 ± 1.2	304 ± 1.3	303 ± 0.5	302 ± 1.0	301 ± 0.4
400	404 ± 1.2	404 ± 1.5	402 ± 0.7	402 ± 1.0	402 ± 0.5
500	504 ± 2.1	504 ± 1.9	503 ± 0.9	502 ± 1.7	502 ± 0.8
600	605 ± 2.4	604 ± 2.4	602 ± 0.8	601 ± 2.1	601 ± 0.7
700	705 ± 4.6	702 ± 2.5	702 ± 1.6	701 ± 1.8	701 ± 1.4
800	806 ± 6.4	804 ± 3.4	803 ± 3.1	802 ± 3.4	802 ± 2.8
900	906 ± 7.2	904 ± 4.0	901 ± 2.3	901 ± 2.4	901 ± 1.9
1,000	1,011 ± 8.8	1,004 ± 4.2	1,001 ± 2.9	1,001 ± 4.1	1,001 ± 2.4
1,200	1,211 ± 15.5	1,205 ± 4.5	1,200 ± 2.4	1,201 ± 6.3	1,200 ± 5.1
1,500	1,512 ± 13.3	1,506 ± 5.6	1,501 ± 0.5	1,499 ± 9.0	1,501 ± 0.5
2,000	2,020 ± 22.5	2,004 ± 7.0	2,001 ± 0.5	1,999 ± 4.2	2,000 ± 0.5
3,000	3,023 ± 19.6	3,023 ± 10.7	2,998 ± 7.2	3,005 ± 10.8	3,001 ± 0.5

Figure 5. HS NGS DNA Ladder analyzed on the 5200 Fragment Analyzer system with the HS NGS Fragment kit. (A) Diluted with 100 mM NaCl (red) and the recommended 1× TE buffer (black). (B) Table displaying average size (bp) and standard deviation. High salt concentrations cause a small increase in sizing. n = 6; LM = lower marker; UM = upper marker.

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# Quality Metrics for Nucleic Acids with the Agilent Fragment Analyzer and Femto Pulse Systems

## Authors

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## Abstract

Assessment of nucleic acid integrity is essential to the success of many downstream applications. The Agilent 5200 Fragment Analyzer system and the Agilent Femto Pulse system offer quick and easy user-friendly metrics for evaluating DNA, RNA, and genomic DNA quality and integrity. The versatility of these quality metrics allows them to be applied to various DNA and RNA samples from different organisms, extraction methods, and tissue types, including extremely degraded formalin-fixed, paraffin-embedded (FFPE) samples.

## Introduction

To assess the quality and integrity of DNA and RNA, four quality metrics are used for the Agilent 5200 Fragment Analyzer system: DQN, GQN, RQN, and DV<sub>200</sub>. These metrics have been published in numerous articles and incorporated in protocols and literature by Illumina, PacBio, and Bionano Genomics.

## 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. An Agilent Femto Pulse system was also used, with an Agilent Genomic DNA 165 kb kit (p/n FP-1002-0275) and an Agilent HS RNA kit (15 nt) (p/n DNF-472).

## Additional Content

Detect low-concentration samples down to the single cell with the unprecedented sensitivity and precision of the Agilent Femto Pulse system.

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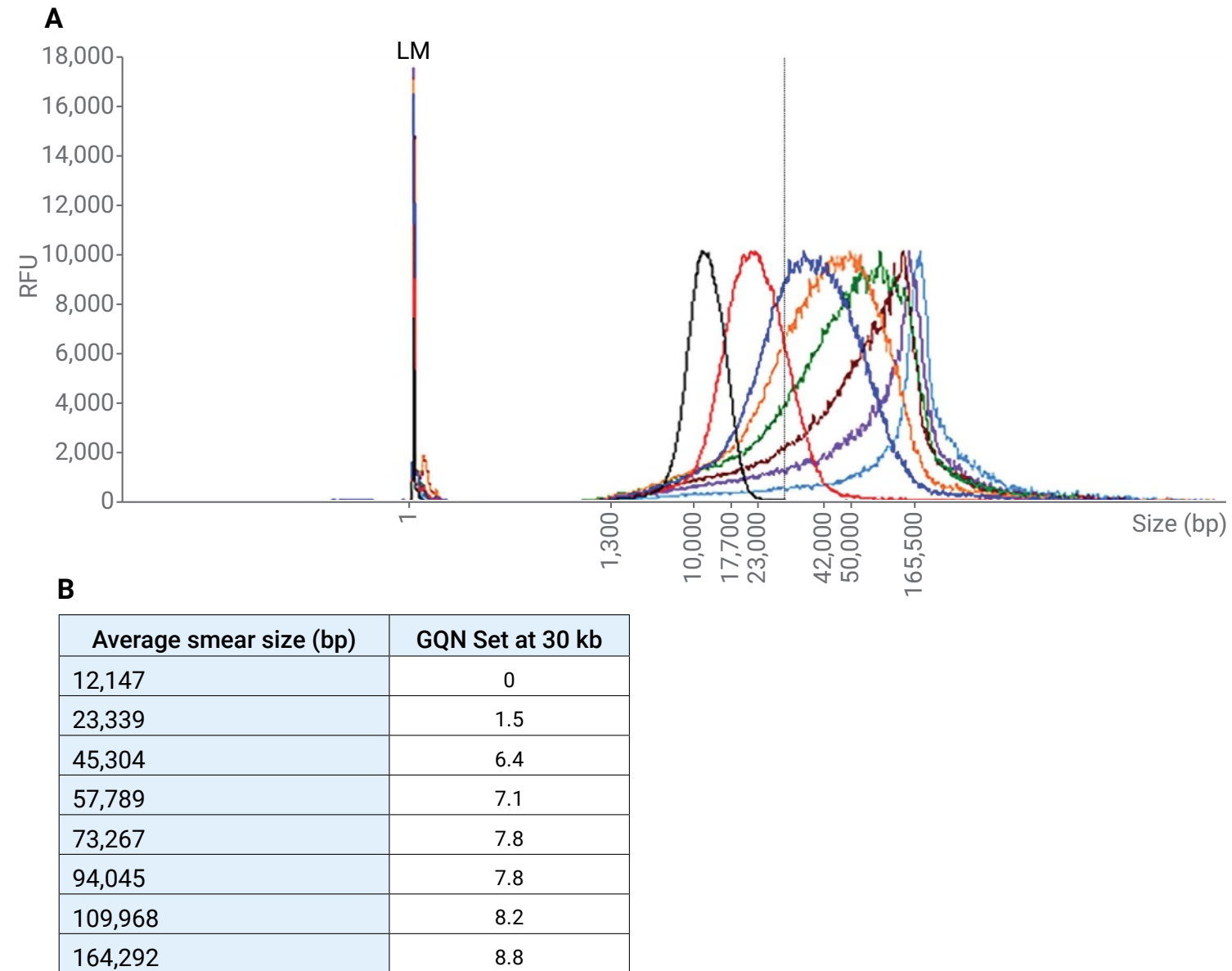
**Results and discussion**

**DQN and GQN**

Agilent designed the DNA quality number (DQN) and the genomic quality number (GQN) for use in ProSize data analysis software to allow for easy analysis of sheared DNA and genomic DNA (gDNA) quality, respectively. The user defines a size threshold they deem appropriate for their specific application. ProSize then calculates a DQN or GQN value based on the fraction of the total measured concentration of the sample that lies above the specified size threshold. The DQN and GQN scores samples on a scale of 0 to 10. 0 indicates that none of the sample exceeds the threshold and 10 indicates 100 % of the sample lies above the threshold value. DNA size can vary due to numerous factors, such as: origin of organism, extraction methods, tissue type, and sample handling. Therefore, the ability to define the size threshold gives the user the advantage of defining quality DNA for their specific application.

GQN is used specifically for genomic DNA samples separated on the 5200 Fragment Analyzer system with the Agilent Genomic DNA 50 kb kit (p/n DNF-467-0500) and Agilent HS Genomic DNA 50 kb kit (p/n DNF-468-0500) and on the Femto Pulse system with the Agilent Genomic DNA 165 kb kit<sup>1</sup>. The DQN is used for all other DNA sample types and was created for samples that use a reagent kit with both a lower and upper marker.

As seen in Figure 1, sheared gDNA samples from PacBio of varying sizes were separated on the



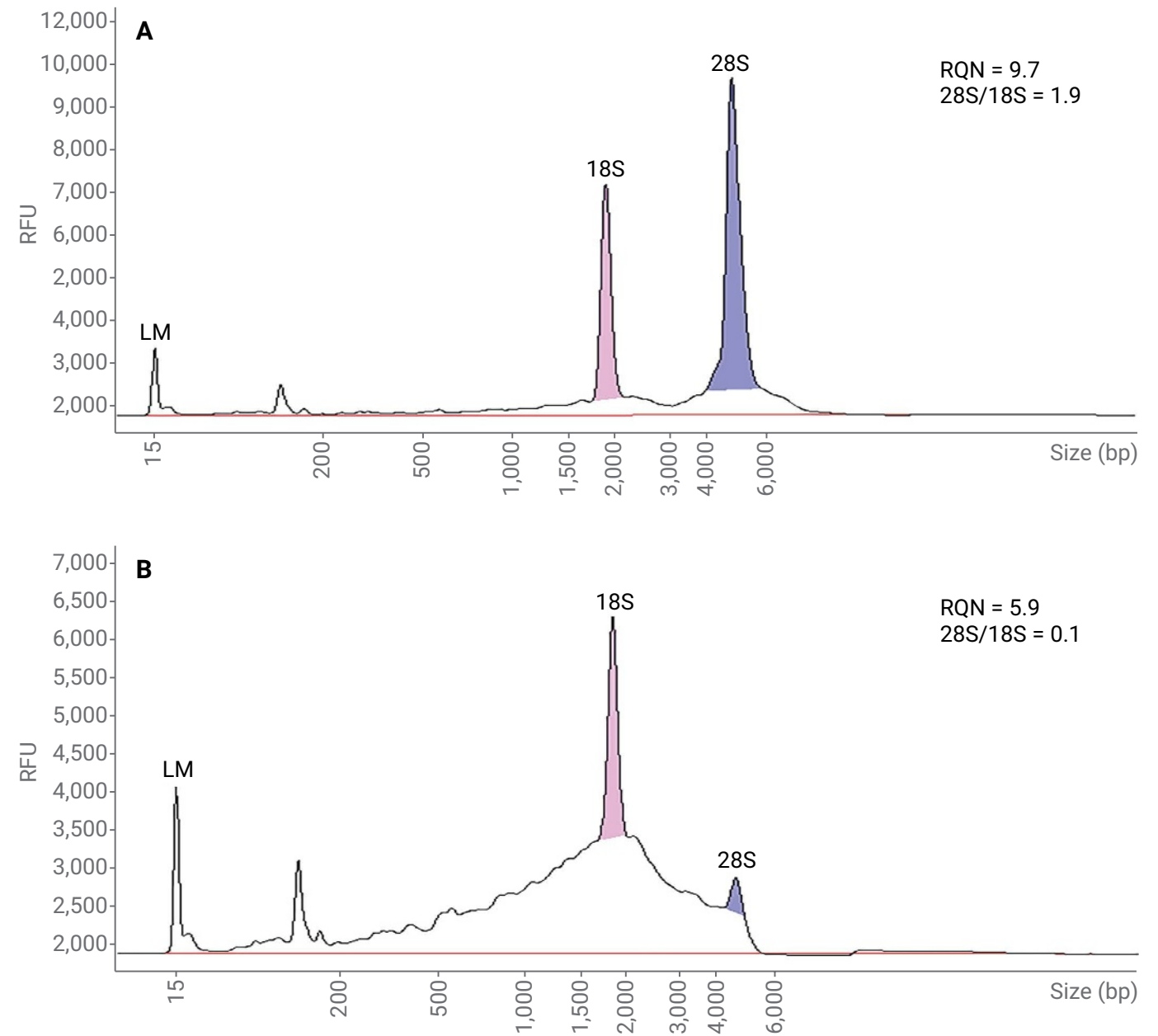
**Figure 1. Sheared gDNA from PacBio separated on the Agilent Femto Pulse system with the Agilent Genomic DNA 165 kb kit. (A) Separations on the electropherogram (B) Average smear size, and GQN<sub>30 kb</sub>, n = 2, LM = lower marker.**

Femto Pulse system with the Agilent Genomic DNA 165 kb kit. The size threshold for these samples was set at 30,000 bp and reported a lower GQN for the smaller sized samples compared to the larger sized gDNA, as expected due to the varying size. The DQN and GQN values allow for fast and easy assessment of the quality of any type of DNA sample.

As a dynamic quality metric, the GQN brings objective analysis to the quality assessment of formalin-fixed, paraffin-embedded (FFPE) gDNA samples. FFPE DNA undergoes substantial chemical modifications during formalin fixation, including cross-linking and fragmentation. Utilizing the 5200 Fragment Analyzer system, Illumina developed three guidelines to follow based on the GQN value when preparing an FFPE library: (1) minimal DNA input into library preparation; (2) number of PCR cycles; and (3) the amount of library used in enrichment<sup>2</sup>. Successful and reliable library preparations and sequencing results can be achieved with the GQN metrics.

### RQN

ProSize uses the RNA quality number (RQN) as a quality metric for total RNA samples. ProSize considers the entire electropherogram including the 5S and fast region where the small RNA separates, as well as the small and large ribosomal peaks, the baseline resolution between the peaks, the ratio of the small and large ribosomal peaks, and the degradation in front of the small ribosomal peak when calculating the RQN. The 5200 Fragment Analyzer system can easily distinguish between small RNA fragments and degradation in the small RNA region, enabling ProSize to assign an accurate RQN. The RQN is based on a scale from 1 to 10 where 1 represents completely degraded total RNA and 10 represents intact total RNA. Universal mouse reference total RNA was separated on the 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt) (Figure 2). Freshly prepared total RNA had a ribosomal ratio of 1.9 and an RQN of 9.7, while the same sample subjected to 70 °C for 10 minutes reported a ribosomal



**Figure 2. Universal mouse reference total RNA separated on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt). (A) 0 minutes at 70 °C, RQN = 9.7. (B) 10 minutes at 70 °C, RQN = 5.9. LM = lower marker.**

ratio of 0.1 and an RQN of 5.9. A large RQN value indicates higher quality RNA with minimal degradation in the sample. The concentration, RQN, and ribosomal ratio are automatically reported in ProSize, allowing for easy evaluation of total RNA quality.

Correlation work demonstrating that the RQN is equivalent to the RNA integrity number (RIN) from the Bioanalyzer was published in 2013<sup>3</sup>. Several peer reviewed publications have since stated that the RQN provided by the 5200 Fragment Analyzer system and the RIN provided by the Agilent 2100 Bioanalyzer system are equivalent indicators of RNA quality<sup>4,5</sup>. Both papers used the 5200 Fragment Analyzer system for assessing RNA quality and presented several electropherograms as examples. Application of the RQN can be found in the Application Note: *Plant RNA Degradation Detection with the Agilent 5200 Fragment Analyzer System*<sup>6</sup>.

### DV<sub>200</sub> metric

FFPE RNA samples are difficult to use, as degradation due to fixation and storage conditions is often quite extensive. It is important to evaluate the quality of each FFPE RNA sample before proceeding with library preparation to eliminate highly degraded samples containing RNA fragments smaller than the optimal size range. Although RQN values are reliable metrics for evaluating the quality of RNA isolated from fresh tissue, it is not a definitive measure of RNA quality

from FFPE samples. To solve this problem, Illumina used the Fragment Analyzer system to develop the DV<sub>200</sub> metric<sup>7</sup>, which calculates the percentage of RNA fragments greater than 200 nucleotides in size. The DV<sub>200</sub> metric is used to determine the minimal RNA input required for successful library preparation and reproducible results. Given the strong correlation between DV<sub>200</sub> values and library yield, the DV<sub>200</sub> metric is ideal for assessing FFPE RNA quality prior to library construction<sup>8</sup>.

### Conclusion

The DQN, GQN, RQN, and DV<sub>200</sub> developed for the 5200 Fragment Analyzer system and Femto Pulse system provide reliable assessment of nucleic acid quality and integrity. The 5200 Fragment Analyzer system provides consistent quantification, sizing, and quality assessment for nucleic acids under 60 kb, while the Femto Pulse system is the only instrument capable of separating and sizing gDNA over 60 kb in as little as 70 minutes. The Femto Pulse system also has the added benefit of extreme sensitivity, with the ability to detect a single cell amount of gDNA. ■

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## NGS Library QC Steps to Ensure Successful Sequencing

*GEN interview with Kyle Luttgahrm, PhD, Product Manager, Parallel CE Instruments, Diagnostics and Genomics Group, Biomolecular Analysis Division, Agilent*

**GEN:** Why is NGS Library QC so important for successful sequencing?

**KL:** Quality control ensures a complete understanding of the starting material, the success of library preparation steps, and the quality of the final library. By having robust quality control procedures, you can be confident in your library preparation prior to flow cell loading. A quality sample is based upon the sample type and application. DNA perfectly suited for short-read sequencing applications may be considered of very low quality for long-read applications. Additionally, a high quality FFPE sample would be considered a highly degraded sample from most other sources.

Electrophoretic analysis of samples lets you decide if the starting material is suitable for individualized applications. When you start a sequencing project it is important to understand the quality of the raw material



**Kyle Luttgahrm, PhD**

as this impacts raw material processes such as shearing. By completing quality control steps throughout the library preparation process proper removal of impurities such as adapter dimers and primer dimers is ensured ultimately allowing for the creation of a symmetrical, artifact-free library for sequencing.

When sequencing library pools it is important that you have equal molar concentrations of each library for proper flow cell loading. This ensures that all your libraries are equally represented. To calculate the molarity, you must determine both the average size and concentration of your samples. This can be

easily accomplished using automated electrophoresis instruments. Once these values are determined, the molarity can be calculated, allowing for equal molar pooling and proper flow cell loading. Due to these factors, all major sequencing instrument providers and library preparation kit manufacturers recommend completing QC steps on the starting raw materials, throughout the library preparation process, all the way to the final library.

**GEN:** I hear there are quality metrics that can help identify samples. What are they and how are they used?

**KL:** Quality metrics provide an objective look at your data. They remove the individual scientist's biases regarding whether or not it is a quality sample. Raw RNA material is where quality metrics are most widely used. Agilent has developed quality metrics for all of our automated electrophoresis instruments, including the RNA Integrity Number (RIN) for the Bioanalyzer system, the RNA Integrity Number Equivalent (RIN<sup>e</sup>) for the TapeStation systems, and RNA Quality Number (RQN) for the Fragment Analyzer and Femto Pulse systems. Each provides a sample a score based on a scale of 1-10, with 10 being very intact RNA and 1 being highly degraded RNA. Due to the difference in the technology used, each system uses a unique algorithm to calculate the RNA quality score; however, they all

correlate strongly and can be used interchangeably.

We also have a couple of quality metrics for genomic DNA (gDNA). The DNA Integrity Number (DIN) can be found on the TapeStation, and the Genomic Quality Number (GQN) on the Fragment Analyzer and Femto Pulse systems. Similar to the RNA metrics, the DIN automatically rates sample quality on a 1 to 10 scale, with 1 being highly degraded and 10 being highly intact gDNA. The GQN also uses a 1 to 10 scale, but is based on a user-defined threshold. Once a threshold is set, the GQN tells you the percent of the DNA that is larger than that threshold, thereby allowing the user to determine the size of gDNA that is considered to be of good quality based upon individual needs and library preparations.

**GEN:** What are the key trends you are seeing in next-generation sequencing?

**KL:** Long-read sequencing is becoming more prevalent. With the increase in read length, it is important to understand how intact the starting genomic DNA is and to perform multiple quality control steps throughout library preparation to ensure that the integrity of the high molecular weight DNA is maintained. Preparation of long-read libraries, such as PacBio, can be time consuming due to these necessary quality control steps which have traditionally been done using agarose pulsed-field gel electrophoresis. The Agilent Femto

Pulse system streamlines the analysis of high molecular weight gDNA by automating pulsed-field separations with sizing through 165 kb in as little as 1.5 hours.

**GEN:** Why is proper sampling, handling, and mixing important for accurate library QC?

**KL:** Proper sampling, handling, and mixing ensures that the results of the quality control analysis are indicative of the sample itself. First, it is important that you are storing your own samples in appropriate conditions. For example, the use of appropriate containers, such as LoBind tubes, can help preserve sample integrity.



Photo by Tim Menzel

Additionally, storage temperature should be taken into consideration. If you are handling high molecular weight DNA it is important that you minimize the number of freeze/thaw cycles as this can contribute to faster degradation. We recommend customers aliquot their high molecular weight gDNA prior to storage. Regardless of sample type, it is important that you maintain aseptic techniques so that you are not introducing any RNases or DNases into your sample.

Second, it is important that you handle the quality control kit reagents properly. Mishandling of kit reagents can lead to degradation of kit components which will adversely affect the quality control analysis. Handling of kit components according to the manufacturer's guidelines ensures that the results from the quality control analysis are accurate representations of your sample.

Finally, how you mix your sample prior to quality control analysis can also drastically influence the results. It is important to mix both your sample and the manufactured supplied reagents according to manufacturer's instructions prior to use. If the provided reagents are to be mixed with your sample, it is important that this is done to manufacturer's specifications. This ensures that when either you or the quality control instrument is sampling an aliquot, that aliquot is representative of the entire sample. Without proper mixing, you can get improper sampling, which will lead to inconsistent results.

**GEN: The quality of genomic DNA can vary widely. How do different extraction methods impact quality and DNA size?**

**KL:** Different kits and protocols can drastically influence the size of the extracted gDNA. Depending on your application, different initial gDNA sizes are required. For example, a long-read sequencing application requires significantly higher molecular weight gDNA than short-read sequencing. By screening your extracted gDNA you ensure that the kit/protocol used is supplying gDNA that is appropriate for your application. We have screened several commercially available kits here at Agilent and have found that they can vary widely in the size of gDNA extracted, even with samples from the same source. Surprisingly, a phenol chloroform extraction consistently provides the highest molecular weight gDNA compared to any other extraction method that we have tried.

Different tissue types can also impact the extracted gDNA size distribution. There are certain cancer cell lines that consistently give higher-quality, higher molecular weight extractions than others. Why this is the case is still an open question. It is important to understand that even if you are using a kit or method that you expect will provide the highest molecular weight DNA possible, that you still may end up with lower molecular weight DNA simply due to the sample type and how that sample was handled prior to you

extracting the gDNA. Utilizing QC steps ensures that your extracted material is suitable for your application.

Regardless of the extraction method used and the sample type you are extracting from, how you handle the extracted gDNA can drastically influence your results. For instance, high molecular weight DNA, if not handled carefully and properly, can be sheared simply by pipetting. We strongly recommend our customers use wide-bore pipet tips when handling high molecular weight gDNA. Since the average gDNA size can impact library preparation protocols, in particular shearing times, we recommend performing quality control analysis after storage and prior to library prep. Looking at the quality of the gDNA immediately before library preparation ensures that you know the quality of your sample, and alleviates concern that the sample may have changed during storage. Proper quality control of samples ultimately gives you confidence in your sample's suitability for downstream applications. ■

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