Application Note

Genomics

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Best Sizing Practices with the Agilent Fragment Analyzer Systems

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.

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

Equation 2. Precision.

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 (1-6000 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/µL and 4.5 to 600 pg/µL, 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/µL. The Agilent HS NGS DNA Ladder (p/n DNF-396) was diluted with 200, 100, 50, and 25 mM NaCl or 1× 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

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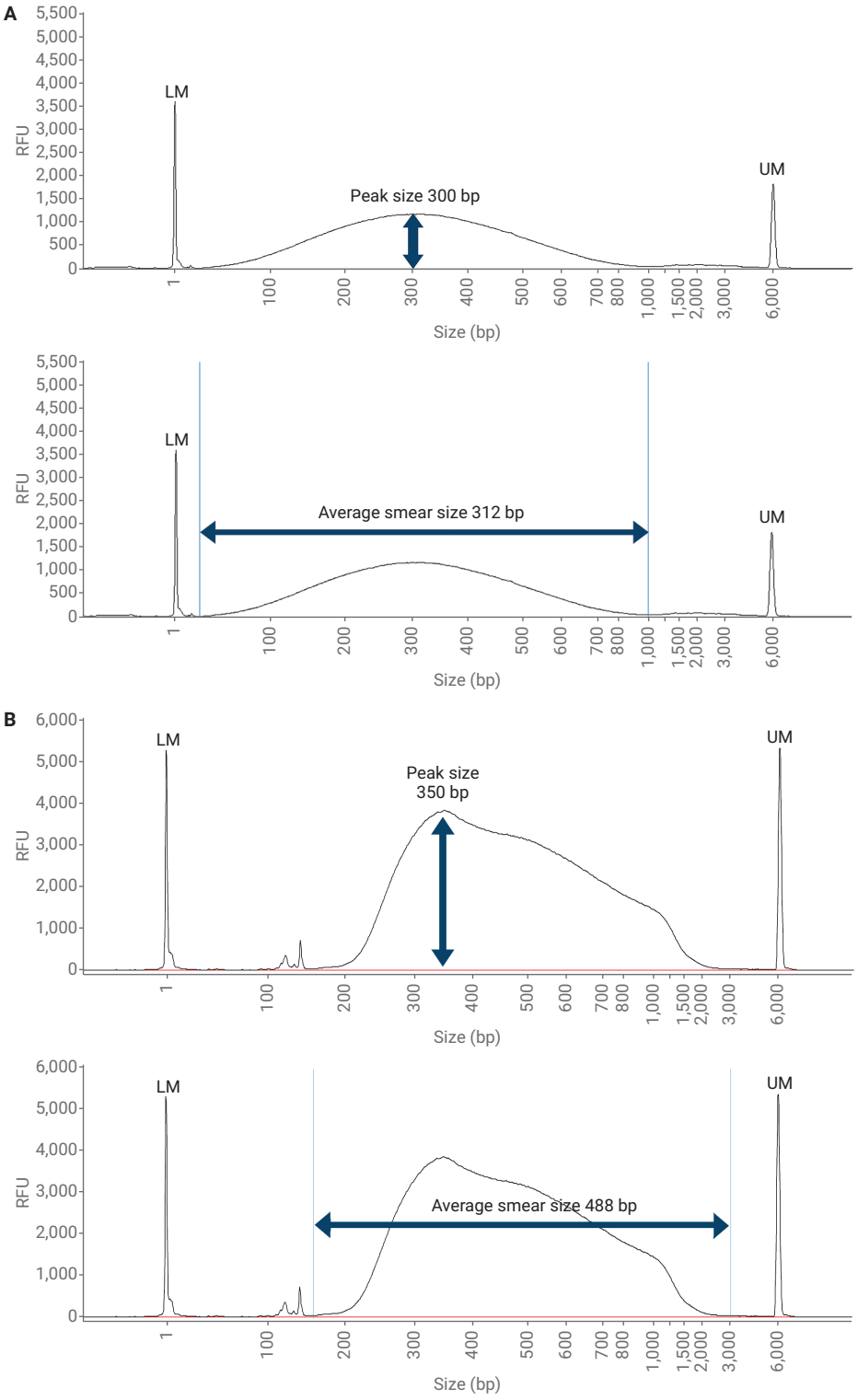


Figure 1. Peak and smear size of DNA libraries analyzed on the 5200 Fragment Analyzer system with the HS NGS Fragment kit (1 to 6000 bp). (A) A DNA library with a bell-shaped curve will report similar peak and smear sizes. (B) A DNA library with a larger distribution of fragments to the right of the 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 ± 1.24 bp and 997 ± 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).

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 (1-6000 bp).
[1] n = 144.

	Sizing over concentration range 4.5 to 600 pg/µL	
	300 bp DNF-474	1,000 bp DNF-474
Average (bp)	299[1]	997[1]
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 %

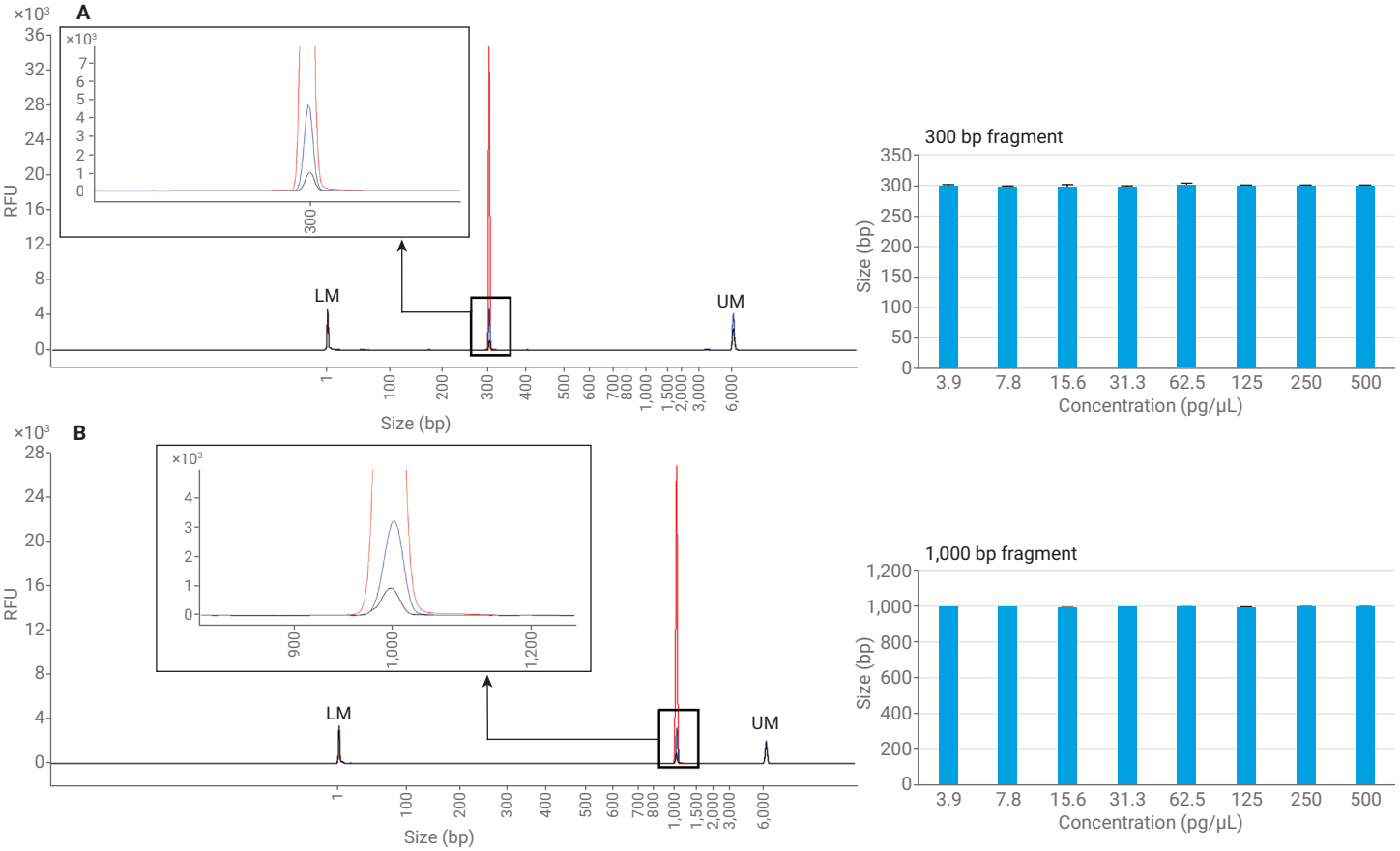


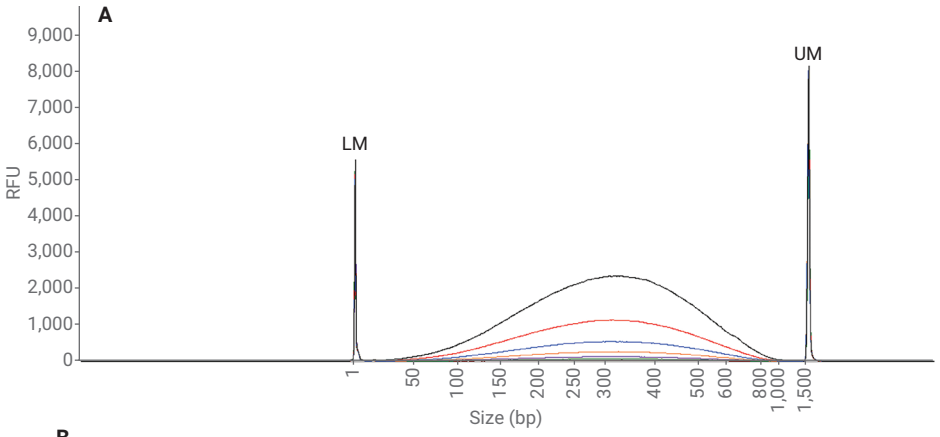
Figure 2. Sizing of 300 and 1,000 bp fragments over a concentration range on the 5200 Fragment Analyzer system with the HS NGS Fragment kit (1 to 6,000 bp). A) 300 bp fragment overlay (15.6, 62.5, and 500 pg/µL) and bar graph (3.9 to 500 pg/µL)n = 18. B) 1,000 bp fragment overlay (15.6, 62.5, and 500 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.

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).

A

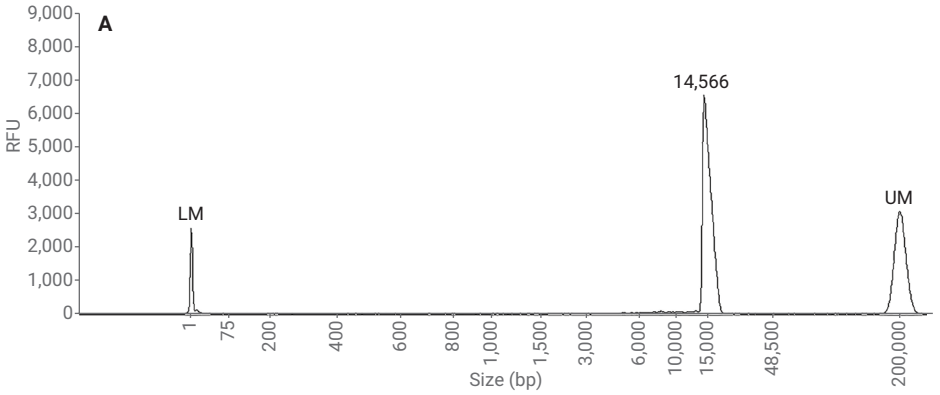


B

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.

A



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 %

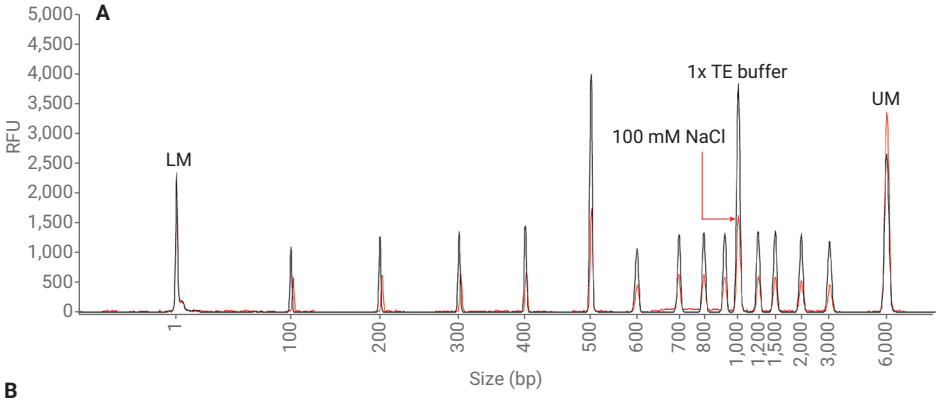
Figure 4. Sizing of 15,000 bp fragment at 600 pg/µL on the 5200 Fragment Analyzer system with the HS Large Fragment 50 kb kit. (A) Electropherogram. (B) Table of 15,000 bp fragment sizing data. n = 3; LM = lower marker; UM = upper marker.

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[1].

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 Fragment 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.

A



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.

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**.

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