Application Note

Genomics

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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.

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 HS 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 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).

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

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 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).

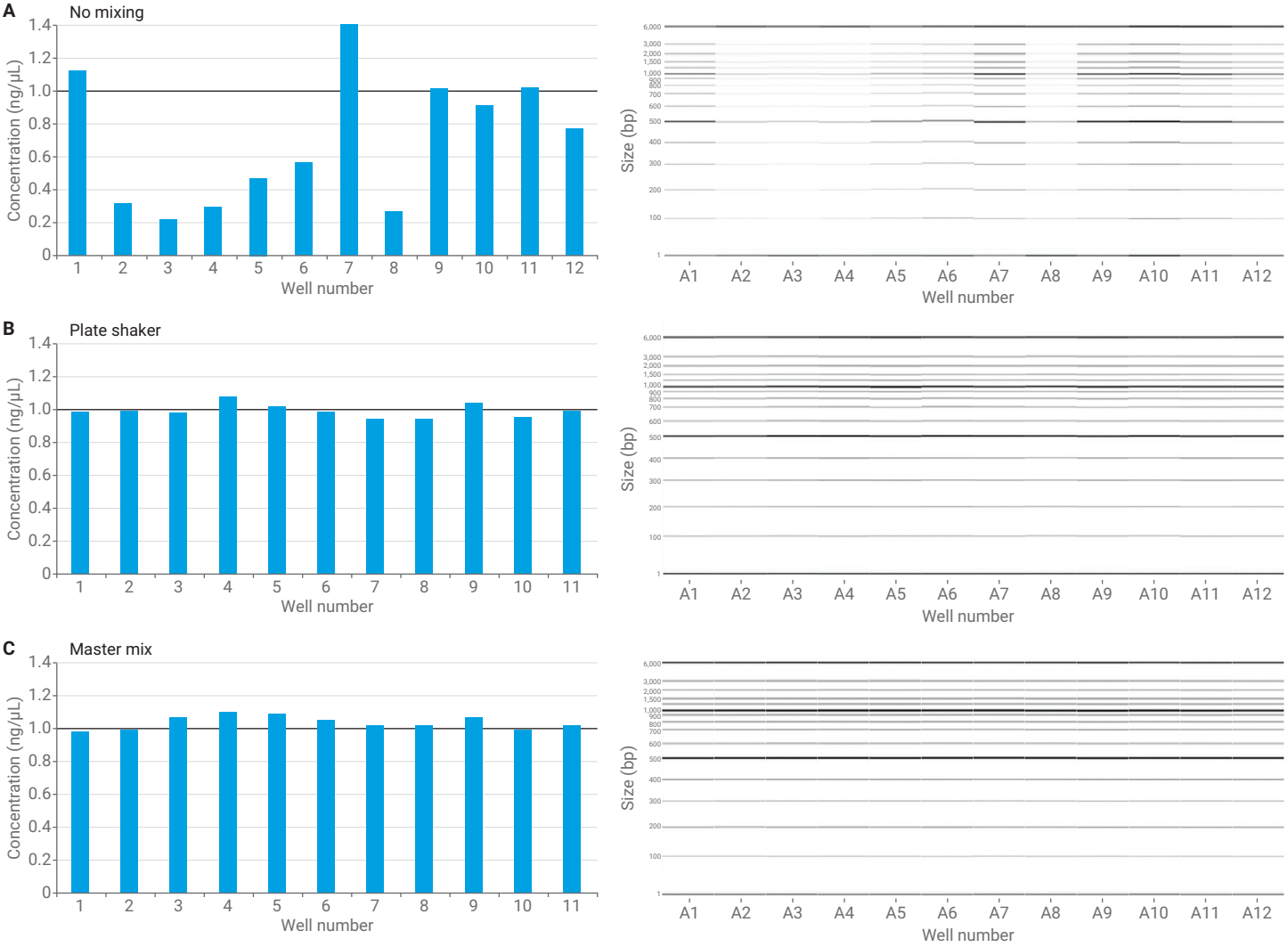

Figure 1. A complete row of Agilent HS NGS DNA Ladder was run on the Agilent 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit (1-6000 bp). Bar graph and gel image. (A) No mixing of the ladder and diluent marker in the plate. (B) Mixing by plate shaker. (C) Master mix of ladder and diluent 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.

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 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.

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[b]	2 µL Pipette mixing 10×[a]	20 µL Pipette mixing 10×[a]	10 µL Electronic pipette mixing 10×[a]	Plate shaker[b]	Master mix vortexed[b]
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 %

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.

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[a]	3.4 %	2.19[b]	3.4 %	2.21[b]	5.9 %
2	5.55[a]	3.8 %	3.07[b]	3.3 %	2.37[b]	1.8 %
3	5.1[a]	0.0 %	3.30[b]	1.6 %	3.22[b]	6.9 %
4	5.2[a]	5.4 %	3.59[b]	6.4 %	2.79[b]	5.8 %
5	197.6[c]	2.6 %	192[c]	2.3 %	181.9[c]	8.1 %
6	100.3[c]	2.1 %	95.9[c]	3.4 %	93.1[c]	7.6 %
7	49.2[c]	1.7 %	46.5[c]	4.6 %	43.9[c]	6.4 %

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 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 3). Reliable analysis of DNA samples is ensured with the correct salt concentration.

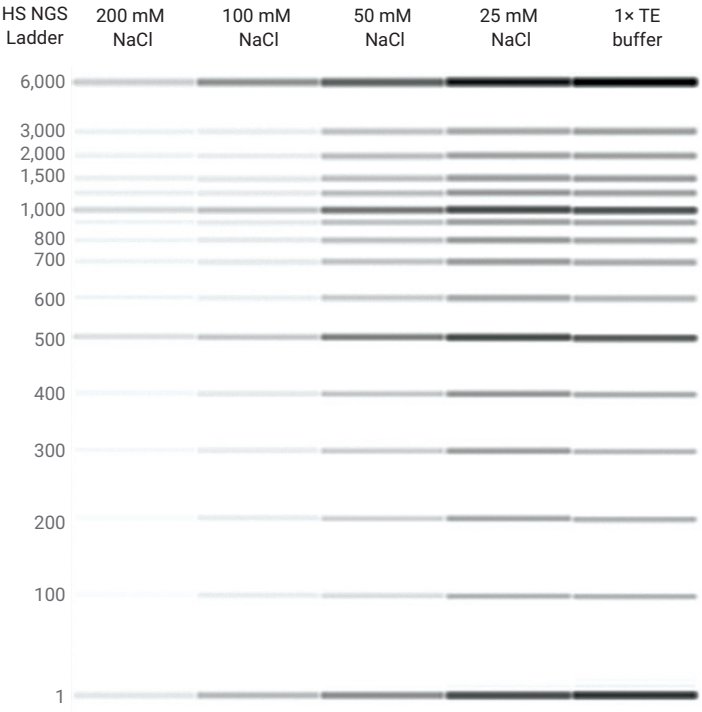

Figure 2. Agilent HS DNA NGS Ladder analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent HS NGS Fragment kit (1-6000 bp). Digital gel image of Agilent HS NGS DNA Ladder diluted in 200, 100, 50, and 25 mM NaCl or 1× TE (10 mM Tris-HCl) buffer.

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.

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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