

Scalable nucleic acid quality assessments for Illumina NGS library preparation

Simultaneous qualification and quantification of nucleic acids with the Agilent Fragment Analyzer system.

Introduction

Assessing nucleic acid quality for library preparation is essential for the success of next-generation sequencing (NGS) applications. Verifying the integrity of nucleic acid samples before library preparation identifies samples likely to produce suboptimal libraries with poor sequencing performance due to degradation, fragmentation, or low purity. Similarly, the accurate assessment of both quality and quantity of prepared libraries can be used to optimize cluster generation during sequencing, resulting in cost- and time savings while maximizing both sequencing data quality and output.

The Agilent Fragment Analyzer systems accelerate nucleic acid analysis workflows and provide a method that has been tested by Illumina for accurate quality assessment with multiple sample types. This application note provides an overview of the quality control (QC) workflow using a Fragment Analyzer system during library preparation (Figure 1) and provides representative data plots for different sample and library types.

The Fragment Analyzer systems

The Fragment Analyzer systems are a proven solution for simultaneous qualification and quantification of DNA and RNA during library preparation for Illumina sequencing workflows. The Fragment Analyzer systems have many enhanced features, including:

- Diverse quantitative sample kit options for genomic DNA (gDNA), NGS libraries, small RNA, total RNA, and messenger RNA (mRNA)
- Seamless, automated switching between applications with two gel input lines
- Scalable array options for parallel CE analysis of 12, 48, or 96 samples

- Multitray format holds three standard 96-well plates for automated analysis of up to 288 samples
- Minimal hands-on time required for instrument setup and sample handling
- High analytical sensitivity detects concentrations as low as 5 pg/μl for fragments and 50 pg/μl for smears
- Ultrahigh-throughput support with the 5400 Fragment Analyzer system, designed to integrate with robotic cells for automated, continuous operation, capable of running over 2400 samples daily without user intervention

Assessing quality of incoming nucleic acids

Assessing the quality of isolated nucleic acid samples will determine if samples are of high enough quality to proceed in the NGS library preparation process.

Nucleic acid quality scores: DNA

The quality of gDNA can be efficiently assessed through calculation of the Genomic Quality Number (GQN). Developed by Agilent for use with the Fragment Analyzer systems, ProSize data analysis software assesses each sample as it relates to a user-defined, application-specific threshold for “good-quality DNA,” and assigns a GQN value between 0 and 10. Values reflect the percentage of DNA above the defined threshold. A low GQN (< 2.5) indicates sheared or degraded DNA. A high GQN (> 9) indicates nondegraded DNA of good quality.¹

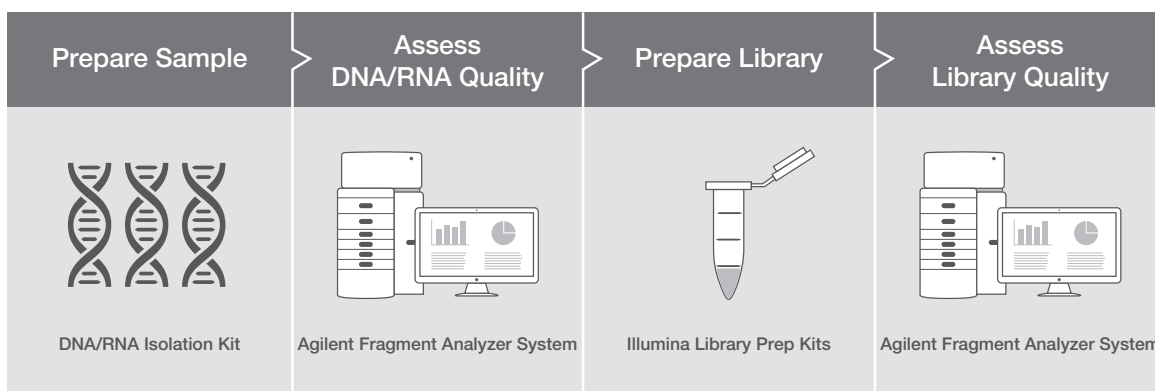


Figure 1: Illumina NGS library preparation workflow—The Fragment Analyzer system is used to assess the quality and quantity of nucleic acids after isolation and after library preparation to enable generation of high-quality sequencing data.

Nucleic acid quality scores: RNA

Two metrics are available for assessing the quality of RNA. ProSize software calculates the RNA Quality Number (RQN), which is equivalent to the RNA Integrity Number (RIN), a broadly accepted metric for total RNA integrity and quality.^{2,3} Similar to RIN values, RQN values range from 1 to 10 and are calculated based on a proprietary algorithm that considers the entire distribution of an RNA electropherogram.³

Although RIN/RQN values are a reliable metric for the quality of RNA isolated from fresh tissue, they are not a sensitive measure of RNA quality from degraded formalin-fixed, paraffin-embedded (FFPE) samples. To solve this problem, Illumina scientists developed the DV₂₀₀ metric, which calculates the percentage of RNA fragments > 200 nucleotides in size. Given the stronger correlation between DV₂₀₀ values and library yield, as compared to RIN values, the DV₂₀₀ metric is ideal for assessing FFPE RNA quality.⁴

Customized methods for the automated DV₂₀₀ metric calculation with the standard and high sensitivity RNA kits can be downloaded from the Agilent website at www.agilent.com/genomics/dv200-calculation.

ProSize data analysis software

The Fragment Analyzer systems use ProSize software to simplify nucleic acid fragment identification and analysis. After data are imported and sizing markers are aligned, fragment sizes and concentrations are calculated automatically with user-defined smear analysis. Data are simultaneously displayed in multiple formats, including a digital gel image (similar to an agarose gel electrophoresis image), an electropherogram, and as a data table. Data are exportable and printable as customizable reports in PDF and CSV file formats.

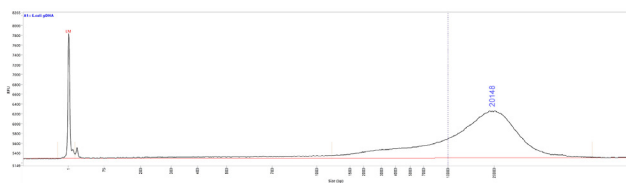
Genomic DNA and FFPE DNA

Quality assessment of gDNA is performed with the Fragment Analyzer system's Genomic DNA 50 kb kit (Agilent, Catalog no. DNF-467), the HS Genomic DNA 50 kb kit (Agilent, Catalog no. DNF-468), or the HS Genomic DNA kit (Agilent, Catalog no. DNF-488). After a single sample dilution step into a 96-well plate, the plate is loaded onto the instrument. Analysis can be completed in less than 40 minutes without further user intervention. The Fragment Analyzer systems detect gDNA smears at concentrations of ≥ 50 pg/ μ l across a wide range of sizes (< 50 bp to 60 kb). ProSize software automatically and accurately determines the size, concentration, and GQN of gDNA samples (Figure 2A).

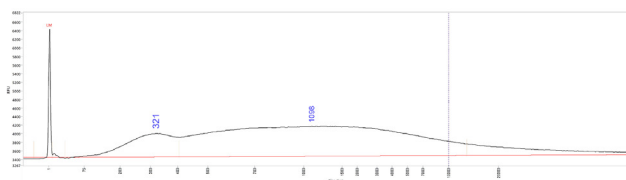
DNA undergoes substantial chemical modifications during formalin fixation, including crosslinking to proteins and degradation into smaller fragments. The quality of DNA isolated from FFPE samples can vary widely due to differences in fixation methods and the age of archival tissue.^{5,6} The Fragment Analyzer systems gDNA analysis kits enable quality assessment of degraded FFPE DNA (Figure 2B and 2C). They deliver GQN values for simplified quality assessment that direct the following:

1. The amount of DNA input into library prep
2. The number of PCR cycles used in the first amplification
3. The amount of library used in enrichment

A. Fresh Tissue



B. FFPE Tissue



C. FFPE Tissue

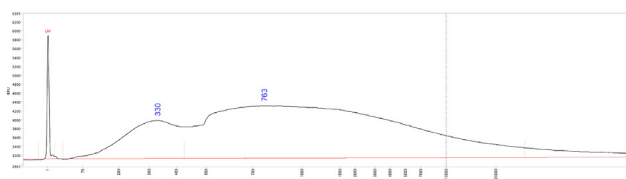


Figure 2: Evaluating quality of gDNA and FFPE DNA—gDNA isolated from (A) fresh and (B and C) FFPE tissue were analyzed on the Agilent Fragment Analyzer system with the HS Genomic DNA kit (DNF-488). A user-defined threshold of 10,000 bp was set (vertical purple lines) for GQN calculations.

Total RNA and FFPE RNA

The Agilent HS RNA kit (15 nt) (Agilent, Catalog no. DNF-472) and RNA kit (15 nt) (Agilent, Catalog no. DNF-471) provide accurate quantification and qualification of total RNA and mRNA samples. Data analysis is simplified with the RNA property summary table in ProSize software.

Each summary includes RNA concentration, the 28S/18S ribosomal RNA (rRNA) ratio, and the RQN (Figure 3A). The rRNA ratio provides a measure of purity of the sample, with ideal values falling between 1.5 and 2.0 for this metric.

Illumina RNA enrichment library prep kits are optimized to provide high-quality RNA sequencing data from degraded FFPE samples, enabling comparison across samples that vary in quality. However, it is important to evaluate the quality of each FFPE sample before proceeding with library preparation to eliminate highly degraded samples containing RNA fragments smaller than the optimal size range for efficient target-capture. The Fragment Analyzer systems provide accurate quantification and DV₂₀₀ metric qualification for FFPE RNA samples (Figure 3B).

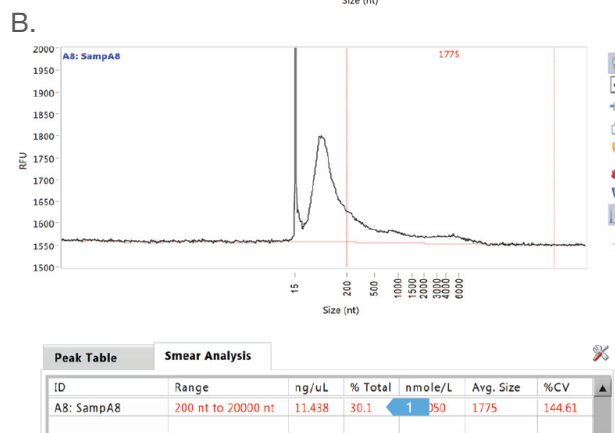
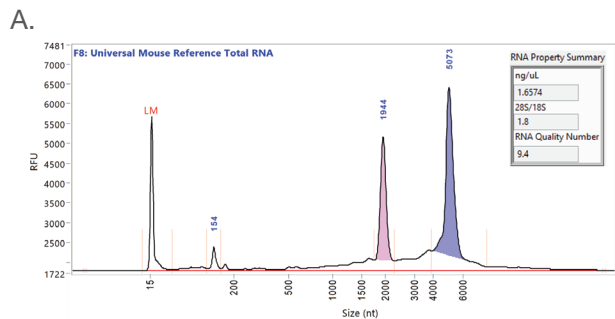


Figure 3: Evaluating quality of total and FFPE RNA—Total RNA isolated from (A) fresh and (B) FFPE tissue were analyzed with the Fragment Analyzer system. ProSize software automatically calculated the DV₂₀₀ metric and displayed the result as the % Total value in the ProSize Smear Analysis table.

Assessing quality of prepared libraries

The Fragment Analyzer systems have been tested for use with most Illumina library preparation kits across many sequencing applications, including whole-genome sequencing, exome sequencing, RNA sequencing, methylation analysis, and targeted panels. Prepared libraries can be quantified and qualified on the Fragment Analyzer systems using the HS NGS Fragment kit (1–6000 bp) (Agilent, Catalog no. DNF-474), the NGS Fragment kit (1–6000 bp) (Agilent, Catalog no. DNF-473), or the HS Small Fragment kit (Agilent, Catalog no. DNF-477).

Assessment of TruSeq Nano and Nextera DNA Flex Libraries

To demonstrate the utility of the Fragment Analyzer systems, a library prepared with the TruSeq Nano Library Prep Kit was quantified and qualified using the HS NGS Fragment kit (Figure 4). The library was determined to have an average smear size of 544 bp and a concentration of 2.15 ng/μL.

The Nextera DNA Flex Library Prep Kit was used to generate a set of libraries from human DNA (NA12878), varying the input from 0.01 ng to 100 ng. Libraries were successfully generated for each input amount by increasing PCR cycle number according to DNA input, with a minimum yield of 100 ng from the 0.01 ng input. All libraries showed approximately the expected size distribution on the Fragment Analyzer system with the HS NGS Fragment Kit (1-6000 bp) (DNF-474) (Figure 5).

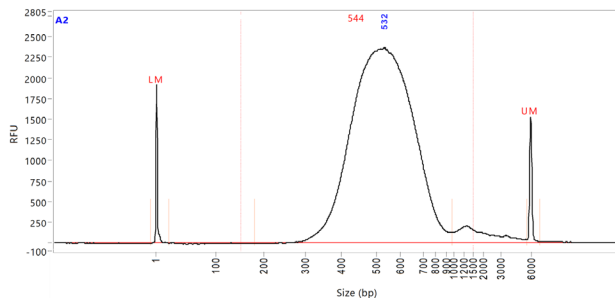


Figure 4: Evaluating quality of a prepared library—A sequencing library prepared with the TruSeq Nano Library Preparation Kit was analyzed with the Fragment Analyzer system.

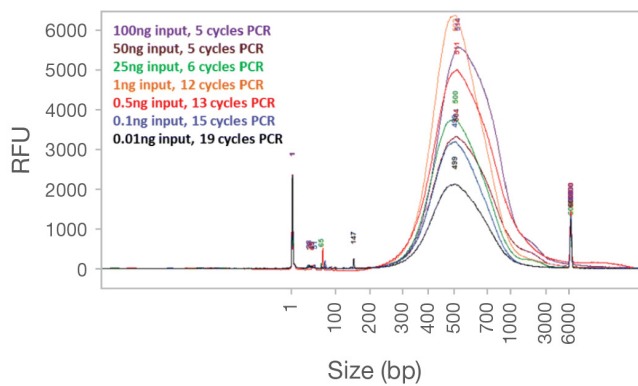


Figure 5: Nextera DNA Flex library preparation from very low input—Library traces for Nextera DNA Flex libraries prepared from DNA input amounts ranging from 0.01 to 100 ng show the expected size distribution for high-quality libraries.

Summary

The Agilent Fragment Analyzer systems are proven platforms for nucleic acid quantification and qualification. The versatility of the Fragment Analyzer systems accommodate low- and high-throughput laboratories with parallel CE analysis of 12, 48, or 96 samples, and automated analysis of up to 288 samples with minimal setup and fast run times. Labs with extremely high sample throughput demands can use the 5400 Fragment Analyzer system within robotic cells for continuous operation and processing of > 2400 samples a day.

Two gel input lines enable the Fragment Analyzer systems to switch seamlessly between multiple applications and analyze different sample types. ProSize data analysis software automatically sizes and quantifies nucleic acids, and provides proven quality metrics for both DNA and RNA isolated from fresh and FFPE tissues. With a growing list of Illumina library prep kits tested for use with the Fragment Analyzer systems, it has become a valuable component of the Illumina library prep QC workflow.

Learn more

To learn more about the Agilent Fragment Analyzer systems, visit www.agilent.com/genomics/fragment-analyzer

To learn more about Illumina NGS library preparation, visit www.illumina.com/techniques/sequencing/NGS-library-prep.html

References

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6. Srinivasan M, Sedmak D, Jewell S. *Effect of fixatives and tissue processing on the content and integrity of nucleic acids*. *Am J Pathol*. 2002;161(6): 1961–1971.