Scalable Nucleic Acid Quality Assessments for Illumina Next-Generation Sequencing Library Prep

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

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 Fragment Analyzer Automated Capillary Electrophoresis (CE) System from Advanced Analytical Technologies, Inc. (AATI) accelerates nucleic acid analysis workflows and provides 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 the Fragment Analyzer during library preparation (Figure 1) and provides representative data plots for different sample and library types.

The Fragment Analyzer

The Fragment Analyzer is a proven solution for simultaneous qualification and quantification of DNA and RNA during library preparation for Illumina sequencing workflows. The Fragment Analyzer has 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
- High throughput support with the Fragment Analyzer *INFINITY*® Automated CE System, designed to integrate with robotic cells for automated, continuous operation, capable of running over 2400 samples daily without user intervention

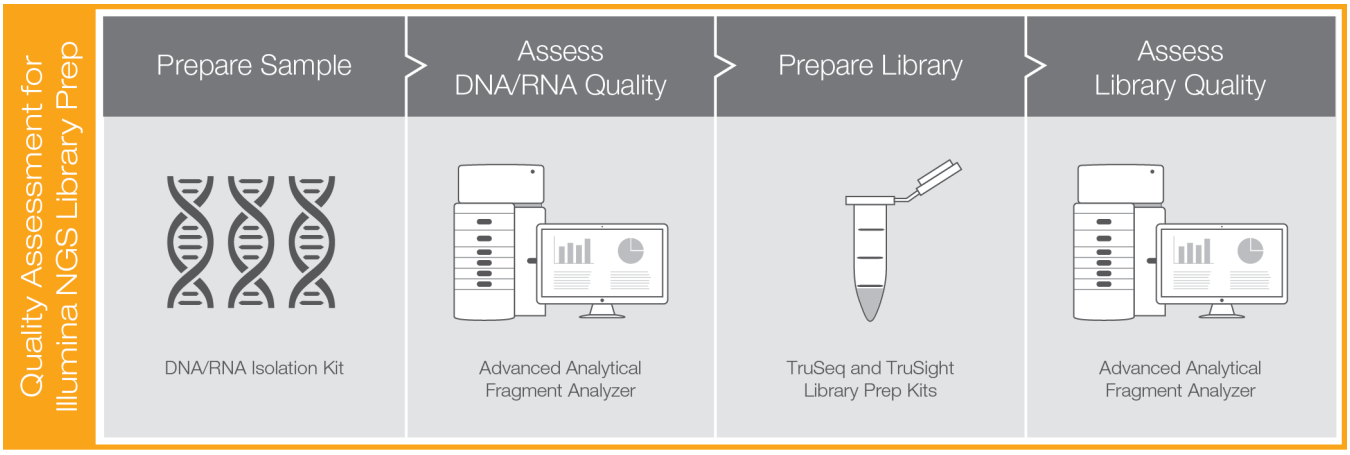


Figure 1: Illumina NGS Library Prep QC Workflow—The Fragment Analyzer is used to assess the quality and quantity of nucleic acids after isolation and after library preparation to enable generation of high-quality sequencing data.

Assessing Quality of Incoming Nucleic Acid Samples

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 AATI for use with the Fragment Analyzer, *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]

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 DV_{200} metric, which calculates the percentage of RNA fragments > 200 nucleotides in size. Given the stronger correlation between DV_{200} values and library yield, as compared to RIN values, the DV_{200} metric is ideal for assessing FFPE RNA quality.[4]

Customized methods for the automated DV_{200} metric calculation with the Standard and High Sensitivity RNA kits can be downloaded from the Advanced Analytical website at www.aati-us.com/instruments/fragment-analyzer/download-dv200-metric/.

The *PROSize* Data Analysis Software

The Fragment Analyzer uses *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 Genomic DNA 50 kb Analysis Kit (AATI Cat. No. DNF-467), the High Sensitivity Genomic DNA 50 kb Analysis Kit (AATI Cat. No. DNF-468), or the High Sensitivity Genomic DNA Analysis Kit (AATI Cat. 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 detects gDNA smears at concentrations of ≥ 50 pg/µl 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 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

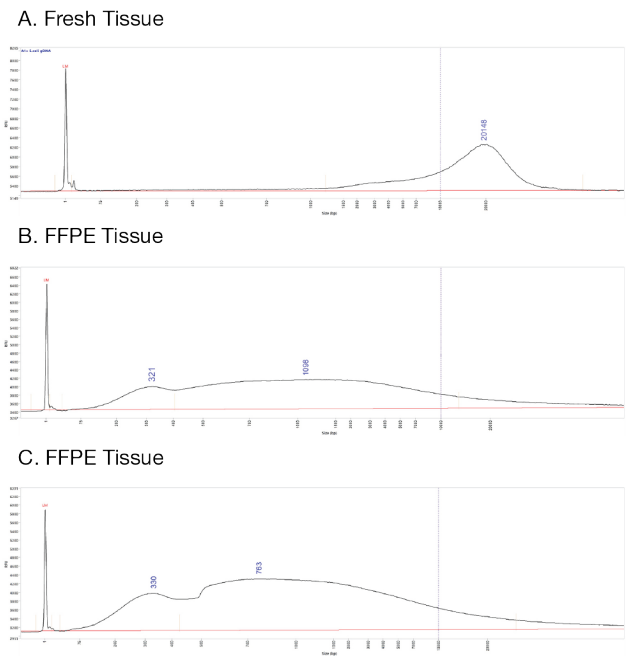


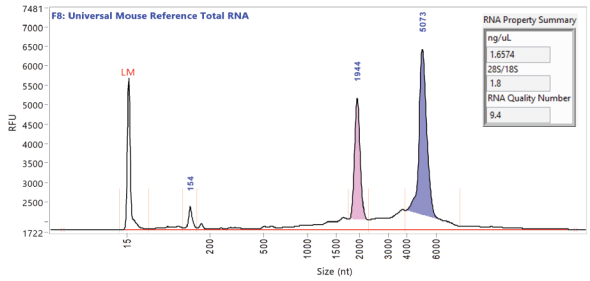
Figure 2: Evaluating Quality of gDNA and FFPE DNA—gDNA isolated from (A) fresh and (B and C) FFPE tissue were analyzed with the Fragment Analyzer High Sensitivity Genomic DNA Analysis Kit. A user-defined threshold of 10,000 bp was set (vertical purple lines) for GQN calculations.

Total RNA and FFPE RNA

The Fragment Analyzer High Sensitivity RNA Analysis Kit, 15 nt (AATI Cat. No. DNF-472) and Standard Sensitivity RNA Analysis Kit, 15 nt (AATI Cat. 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 provides accurate quantification and DV_{200} metric qualification for FFPE RNA samples (Figure 3B).

A.



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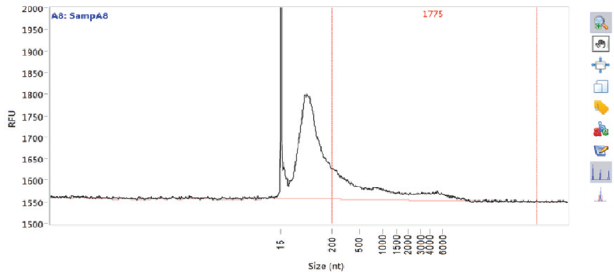

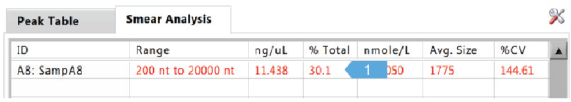

Figure 3: Evaluating Quality of Total and FFPE RNA—Total RNA isolated from (A) fresh and (B) FFPE tissue were analyzed with the Fragment Analyzer. *PROSize* software automatically calculated the DV_{200} metric and displayed the result as the % Total value in the Smear Analysis Table.

Assessing Quality of Prepared Libraries

The Fragment Analyzer has been tested for use across many Illumina TruSight® and TruSeq® library prep kits and applications to date, including whole-genome sequencing, exome sequencing, RNA expression, methylation, amplicon, and targeted panels (Table 1). Prepared libraries can be quantified and qualified on the Fragment Analyzer using the High Sensitivity NGS Fragment Analysis Kit, 1 bp – 6,000 bp (AATI Cat. No. DNF-474), the Standard Sensitivity NGS Fragment Analysis Kit, 1 bp – 6,000 bp (AATI Cat. No. DNF-473), or the High Sensitivity Small Fragment Analysis Kit, 1 bp – 1,500 bp (AATI Cat. No. DNF-477).

Table 1: Illumina Library Prep Kits Tested on the Fragment Analyzer[a]

Application	Illumina Library Prep Kit
Genomic	TruSeq Nano DNA
	TruSeq PCR-Free
Exome	TruSeq Exome
	TruSeq Rapid Exome
Methylation	TruSeq DNA Methylation
ChIP	TruSeq ChIP
Panel/Capture	TruSight Tumor 15
	TruSight Tumor 170
	TruSight One
	TruSight Inherited Disease
	TruSight Cardio
	TruSeq Bovine Parentage
Amplicon	TruSeq Custom Amplicon Low Input
	TruSeq Custom Amplicon 1.5
RNA	TruSight RNA PAN Cancer
	TruSeq Targeted RNA Expression (TREx)
	TruSeq RNA Access
	TruSight RNA Fusion Panel

a. List is current as of the publication date of this application note.

Assessment of a TruSeq Nano Library

To demonstrate the utility of the Fragment Analyzer, a library prepared with the TruSeq Nano Library Prep Kit was quantified and qualified using the High Sensitivity NGS Fragment Analysis Kit, 1 bp – 6000 bp (Figure 4). The library was determined to have an average smear size of 544 bp and a concentration of 2.15 ng/µl.

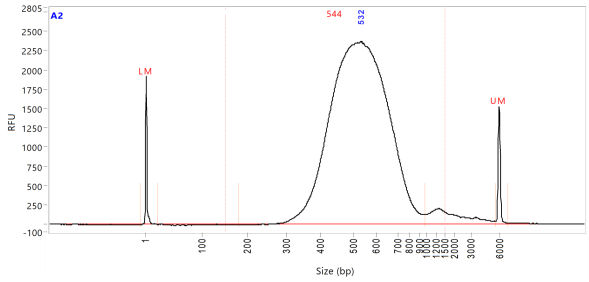

Figure 4: Evaluating Quality of a Prepared Library—A sequencing library prepared with the TruSeq Nano Library Prep Kit was analyzed on the Fragment Analyzer.

Summary

The Fragment Analyzer Automated CE System from Advanced Analytical Technologies, Inc. is a proven platform for nucleic acid quantification and qualification. The versatility of the Fragment Analyzer accommodates 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 Fragment Analyzer *INFINITY* Automated CE System within robotic cells for continuous operation and processing of > 2400 samples a day.

Two gel input lines enable the Fragment Analyzer 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, it has become a valuable component of the Illumina library prep QC workflow.

Learn More

To learn more about the Fragment Analyzer, visit www.aati-us.com/instruments/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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2. Agilent Technologies. (2016). RNA Integrity Number (RIN) – Standardization of RNA Quality Control Publication. PN-5989-1165EN. Accessed March 2017.
3. Wong KS, Pang H. Simplifying HT RNA Quality & Quantity Analysis. *Genet Eng & Biotech News*. 2013;33(2):4688.
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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.

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