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Automated Parallel Capillary Electrophoresis

# Nucleic Acid Analysis for Sample Quality Assessment Using the Agilent Fragment Analyzer Systems

Application Compendium

Agilent Automated Electrophoresis

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Application Compendium

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Recent years have seen the proliferation of genetic and genomic analyses throughout both the research lab and the clinic. The applications of these technologies are as numerous as their methods and include markets such as:

- Pharmaceutical/biopharmaceutical
- Genetic testing labs
- University or Institute core facilities
- Sequencing service providers
- Biobanks and repositories
- Synthetic bio/genome engineering
- Plant, forestry and animal genomic analysis
- Environmental testing
- Clinical research and human disease research
- Microbial/infectious disease research

No matter the application, getting the most from your research requires a robust workflow. The integration of sample assessment and quality control (QC) checkpoints into workflows helps accomplish this by providing accurate information about nucleic acid samples, aiding in workflow optimization and minimizing the use of unfit samples in downstream applications.

The Agilent Fragment Analyzer systems are parallel capillary electrophoresis instruments that were designed for reliable and accurate nucleic acid sample assessment. A broad range of application kits are available, allowing you to easily size, qualify, and quantify both DNA and RNA samples. With its unique design and intuitive features, common QC bottlenecks are eliminated, and lab efficiency is increased with the ability to load multiple gels for both DNA and RNA applications. There are three models to choose from with varying throughputs to match your lab's needs.

Common analysis workflows and samples that leverage the Fragment Analyzer systems for quality assessment include:

- Genomic DNA (gDNA)
- Cell-free DNA (cfDNA)
- Formalin-fixed paraffin-embedded (FFPE) DNA
- Next-generation sequencing (NGS) library preparation
- Genotyping
- PCR amplicons
- Simple sequence repeats (SSR)/ microsatellite amplicons
- Restriction digest analysis
- Plasmid DNA
- CRISPR-TILLING mutation detection
- CRISPR guide RNA
- Polyadenylated IVT RNA
- Total RNA
- mRNA
- IVT RNA
- FFPE RNA
- Small RNAs (including microRNAs)

To demonstrate the benefits of sample QC in these workflows, we compiled this compendium of application notes written by Agilent scientists. We also briefly describe capillary electrophoresis technology and the Fragment Analyzer system product portfolio. This application compendium demonstrates the advantages offered by sample assessment and how easily it can be incorporated into virtually any genomics workflow.

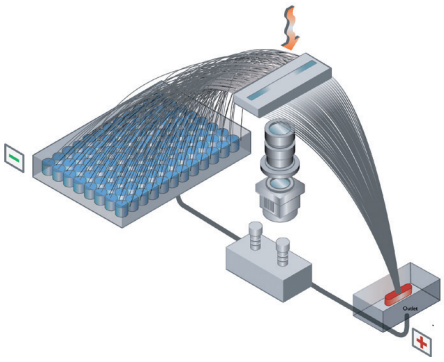
# Agilent Fragment Analyzer Systems

**The Fragment Analyzer systems utilize automated parallel capillary electrophoresis to provide reliable QC for a broad variety of sample types and applications, including NGS libraries, RNA samples, PCR amplicons, and CRISPR workflows.**

The **Fragment Analyzer systems** break through analytic bottlenecks by providing a sample quality assessment tool that easily integrates with key genetic analysis workflows, giving researchers the results they need, when they need them. Automated parallel capillary electrophoresis enables analysis of multiple samples at once without researcher intervention. The ability to accommodate two different gel matrices allows for unattended and consecutive analysis of multiple reagent kits.

The **Fragment Analyzer systems** are indispensable for sample QC because of their many benefits, including:

- Flexible, interchangeable arrays allow for adjustable throughputs and applications to fit the changing needs of the laboratory
- Minimal sample concentration requirements enable researchers to conserve precious samples for further analysis



## Capillary electrophoresis

The capillary array is the basis of the Fragment Analyzer systems, as it can reliably separate both DNA and RNA samples, easily switching between applications. The arrays are available in three different lengths (short, ultrashort, and long) to allow the user to prioritize separation resolution or time, depending on workflow needs. A shorter capillary array offers faster run times, while longer capillary arrays offer improved separation resolution.

### Principles of capillary electrophoresis:

1. Individual capillaries are filled with gel that serves as the separation matrix
2. Samples are voltage-injected into the capillaries, and each sample moves through an individual capillary in a size-dependent manner
3. As the fragments pass the detection window, a sensitive charged coupled device (CCD) detector captures the size and concentration level of the sample
4. The resulting series of images are merged together to produce a high-resolution electropherogram across the entire sizing range

# Fragment Analyzer Models

With three different models, the **Fragment Analyzer systems** are easily adaptable to the workflow of any lab:

The **5200 Fragment Analyzer and 5300 Fragment Analyzer systems** provide variability in throughput by using different capillary arrays.

- The **5200 Fragment Analyzer system** uses a 12-capillary array allowing for 12 samples to run at one time
- The **5300 Fragment Analyzer system** offers higher throughput, with the ability to run either a 48- or a 96-capillary array
- Both models have the ability to load up to three 96-well plates and process in any order

The **5400 Fragment Analyzer system** is an ultrahigh-throughput system, enabling the analysis of thousands of samples per day.

- The system utilizes a 96-capillary array and is capable of full integration with most robotic systems
- An application program interface (API) controls the movement of the buffer, waste, and sample drawers, allowing for continuous runs without user intervention
- Integrated software packages allow for remote control of the instrument

*Note: Experiments performed using the 5200 Fragment Analyzer system and can be replicated with comparable results on Agilent 5300 Fragment Analyzer and 5400 Fragment Analyzer systems when using the same array length.*

## Features and benefits of the Fragment Analyzer systems

- With electrophoresis times as short as 15 minutes, no daily array handling, and room-temperature stable reagents, instrument set-up and run time is minimized allowing faster time to results
- 3 bp resolution of fragments approximately 300 bp and smaller provides discrimination of closely sized products giving you confidence in sample composition
- Two orders of magnitude dynamic range enables loading of unknown quantities, eliminating the need to predetermine sample concentration
- Quality metrics for RNA (RNA quality number, RQN) and genomic DNA (genomic DNA quality number, GQN) allow for objective quality assessment aiding nucleic acid sample standardization
- Always-accessible sample drawers permit the addition of samples, even while the instrument is running, eliminating down time
- Ability to re-arrange the sample queue even while the instrument is running lets high priority samples to be run sooner

## Reagent kits for the Fragment Analyzer systems

A broad range of kits are available for the Fragment Analyzer systems, allowing you to easily qualify and quantify both DNA and RNA samples. The diversity of sample types these systems can separate make these instruments ideal for a variety of workflows, including sample QC for NGS library preparation.

The **Small Fragment and NGS kits** facilitate the separation of DNA fragments, smears and NGS libraries, with sizing from 50 to 1,500 bp and 100 to 6,000 bp, respectively. The NGS kit covers a concentration range of 0.1 to 10 ng/µL for fragments and 5 to 100 ng/µL for smears. The HS Small Fragment and HS NGS kits have a smaller concentration range of 5 to 500 pg/µL for fragments and 50 to 5,000 pg/µL for smears. Each of the kits provides accurate quantification and sizing, making them ideal for NGS library preparation workflows.

The **Large Fragment kits** are used for automated qualitative and quantitative analysis for large DNA fragments and smear up to 48,500 kb. Each of the kits has a different input concentration range, specific for fragments or smears. The Large Fragment kits are ideal for reliable QC checkpoints in many different workflows, including long-read NGS, formalin-fixed paraffin-embedded (FFPE) DNA analysis, and restriction digest analysis. By comparing the sizing of sheared and unsheared large DNA samples, the kits provide a reliable evaluation of DNA fragmentation, an essential step in the preparation of large-insert libraries.

The **Genomic DNA kits** were developed for the separation of genomic DNA (gDNA). Automated assessment of gDNA size and integrity is extremely beneficial for QC of samples to be used in long-read and whole genome NGS, metagenomics, and analysis of degraded DNA. A broad sizing range allows accurate and precise sizing of samples through 60 kb. Covering expansive concentration ranges, the HS gDNA 50 kb kit is for samples ranging from 0.3 to 12 ng/µL, while the gDNA 50 kb kit extends from 25 to 250 ng/µL. Easily analyze gDNA samples with a user-defined quality metric (GQN), which allows users to decide what qualifies as good gDNA for their purposes.

The **RNA kits** can be used for analysis of both total RNA and mRNA, including IVT mRNA sizing. Ensuring quality RNA is crucial to many downstream applications, including NGS and gene expression studies. RNA analysis with the RNA kits provides each sample a quality metric, the RQN. Excellent resolution allows for distinction between small RNA and degraded RNA, providing a reliable and accurate RQN score. Specific RNA analysis modes are available for eukaryotic, prokaryotic, plant, and mRNA samples. The RNA kits have a sizing range of 200 to 6,000 nt. The RNA kit (15 nt) covers a concentration range of 5 to 500 ng/µL, while the HS RNA kit is for less concentrated samples, with a range of 50 to 5,000 pg/µL.

The **Small RNA kits** focus on microRNA and small RNA QC analysis, essential for downstream applications such as small RNA NGS library preparation. The kits provide accurate and precise quantification and sizing of small RNA and microRNA, focusing on the narrow range from 15 to 200 nt. Focusing on this small sizing range allows for high-resolution separation and detailed analysis of both the microRNA (10 to 40 nt) and small RNA (40 to 200 nt) regions. A region analysis function automatically calculates the percent microRNA and quantifies the microRNA and small RNA regions.

The **Qualitative DNA kits** provide automated and accurate sizing with relative quantification of DNA fragments. Common uses include the analysis of PCR amplicons, simple sequence repeats (SSRs)/microsatellites, and genotyping. There are six different kits to choose from based on the size of the DNA or PCR fragments, ranging from 35 to 20,000 bp. Specially formulated gels, markers, and ladders are designed for each kit, enabling reliable sizing over a broad range with varying applications. High-resolution kits enable the separation of fragments under 300 bp with a 3 bp difference.

The **Plasmid DNA kit** is used in the analysis of supercoiled and linear plasmid DNA. The ladder provided with this kit is optimized for the accurate sizing and relative quantification of supercoiled plasmids between 2,000 and 10,000 bp. Linearized plasmids can be evaluated for quality and relative quantification; however, only comparative sizing is possible.

Efficient and accurate identification of CRISPR mutation events is a critical component of CRISPR/Cas9 workflows. The **CRISPR Discovery Gel kit** automates the screening of heteroduplex cleavage assays and provides fragment sizing and relative concentration. These important pieces of information are automatically processed by ProSize data analysis software to indicate successful editing events and other crucial information like percent mutated.

# Quality Metrics Overview

High-quality nucleic acids are necessary for successful library preparations and sequencing results. Nucleic acid QC can determine which samples are not suitable for library preparation. Not all extraction methods are the same, resulting in nucleic acids with varying integrity. Nucleic acid samples such as FFPE, ancient samples, and RNA are easily degraded due to chemical fixation, time, temperature, enzyme digestion, and improper handling. If a sample is too far degraded, it will result in poor sequencing results; important coding areas of interest are lost, and gaps appear in the full-length gDNA. Knowing the quality of the input nucleic acid helps provide guidance for changes needed to optimize workflow for applications such as NGS, long read sequencing, microarray, and genotyping.

Quality metrics provide the user with reliable assessment of the integrity of a sample. Users can establish quality metric standards in their workflows by doing degradation studies and comparing the quality metrics scores between the samples. In addition, quality metrics can save time and money by reducing variation between user assessments and misinterpretation of the results, while easily identifying unfit starting material that would lead to poor results. The Fragment Analyzer instruments provide reliable quality metrics for different sample types.  The Agilent quality metrics are widely accepted and referenced in literature. The Fragment Analyzer systems ProSize data analysis software has the quality metrics built in for easy assessment of samples.

## Genomic DNA Quality Number (GQN)

Genomic DNA (gDNA) is easily sheared with routine handling, such as pipetting, mixing and vortexing, and multiple freeze-thaw events. DNA from fresh, frozen, or FFPE tissue can be assessed with the genomic DNA quality number (GQN) on the Fragment Analyzer systems. Agilent designed the GQN to allow for easy analysis of sheared DNA and gDNA quality. The user defines a size threshold they deem appropriate for their specific application. The GQN value is then calculated based on the fraction of the total measured concentration of the sample that lies above the specified size threshold. The GQN scores samples on a scale of 0 to 10. A score of 0 indicates that none of the sample exceeds the threshold and 10 indicates 100% of the sample lies above the threshold value.

## RNA Quality Number (RQN)

Total RNA from fresh or frozen tissue can be assessed with the RNA quality number (RQN). The RQN takes into consideration 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. It is calculated using a scale from 1 to 10. A high RQN indicates highly intact RNA, and a low number, strongly degraded RNA sample. Several studies have been performed demonstrating the equivalences of the Bioanalyzer RIN to the RQN as highlighted in the Analysis of total RNA section.

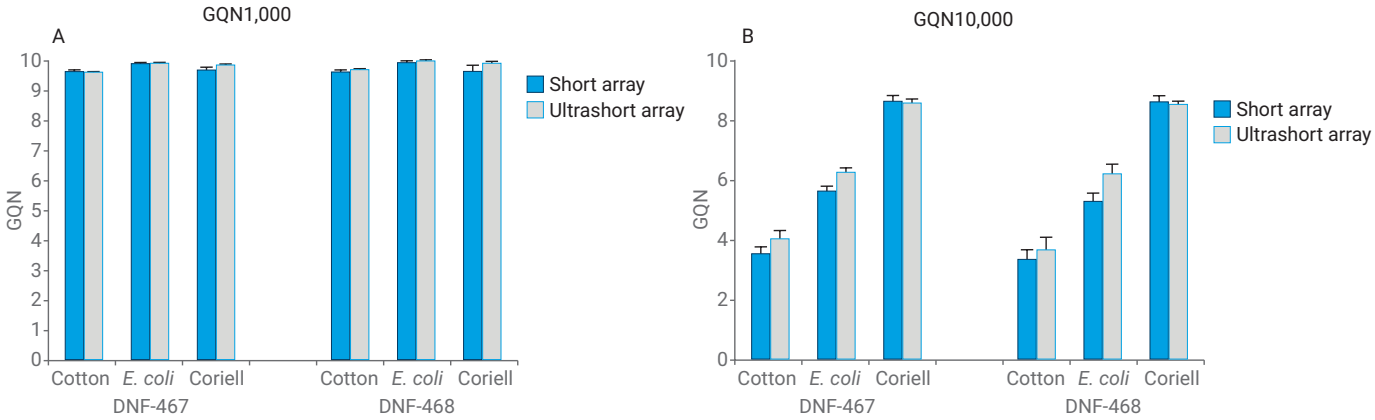
## $DV_{200}$

FFPE RNA samples are challenging to analyze, as degradation due to fixation and storage conditions is often 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 the RQN value is a reliable metric for evaluating the quality of RNA isolated from fresh, frozen, or cell culture tissue, it is not a definitive measure of RNA quality from FFPE samples. To solve this problem, the $DV_{200}$ quality metric was developed for use with the Agilent Automated Electrophoresis systems including the Fragment Analyzer. It calculates the percentage of RNA fragments greater than 200 nucleotides in size. The $DV_{200}$ metric is then used to determine the minimal RNA input required for successful library preparation and reproducible results. Given the strong correlation between $DV_{200}$ values and library yield, the $DV_{200}$ metric is ideal for assessing FFPE RNA quality prior to library construction.

| | Fragment Analyzer Systems Quality Metrics | | |
|---|---|---|---|
| Metric | GQN | RQN | $DV_{200}$ |
| Sample Type | gDNA and FFPE DNA | RNA | FFPE RNA |
| Definition / calculation | Calculated based on the fraction of the total measured concentration of the sample that lies above the specified size threshold, on a scale from 0 to 10. A score of 10 indicates that 100% of the sample is observed above threshold. | Calculated using a scale from 1 to 10. A high RQN indicates highly intact RNA, and a low number, strongly degraded RNA sample. | Calculation of the percentage of RNA fragments greater than 200 nucleotides in size. The $DV_{200}$ metric is then used to determine the minimal RNA input required for successful library preparation and reproducible results. |

# Analysis of DNA

## GQN quality metrics for genomic DNA quality assessment




**Kits:** **Genomic DNA 50 kb kit; HS Genomic DNA 50 kb kit**

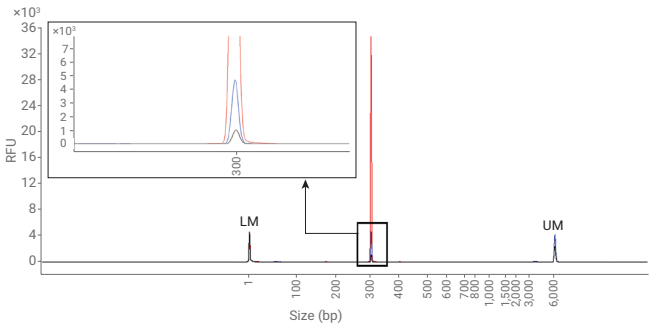
**Abstract:** Quality assessment of nucleic acids is critical to the success of many downstream applications, including NGS. The Fragment Analyzer systems provide quick and easy assessment of gDNA quality and integrity with the GQN. The GQN is commonly used for evaluating the input gDNA material for NGS library preparation. To prepare a successful library, the sample must be of the correct size and of sufficient quality for sequencing. The GQN threshold can be set by the user to reflect the size threshold necessary for their particular requirements. The GQN is given on a scale of 0 to 10, with a higher score indicating that more of the sample exceeds the user-defined threshold. The GQN size threshold was set at 1,000 bp (A) and 10,000 bp (B) for cotton, *E. coli*, and Coriell gDNA samples, demonstrating the GQN flexibility when evaluating the quality of gDNA. The higher 10,000 bp size threshold gave a lower GQN for the smaller sized cotton (3.6) and *E. coli* (5.9) samples compared to Coriell (8.6), as expected due to their varying size. The lower 1,000 bp size threshold reported a similar GQN for all three samples because the majority of the sample lies above the size threshold parameter. The GQN values were consistent between both the Genomic DNA 50 kb kit and the HS Genomic DNA 50 kb kit on both the short and ultrashort arrays for all samples.

**Application note: 5994-0511EN**

# Analysis of DNA

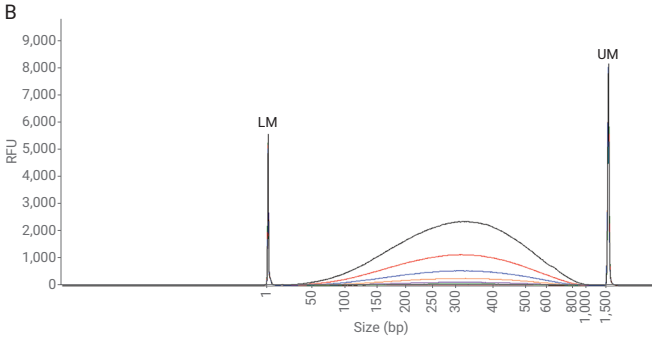
## Best sizing practices

A




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

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 % |

**Kits:** **HS NGS Fragment kit (1-6000 bp); HS Small Fragment kit; HS Large Fragment 50 kb kit**

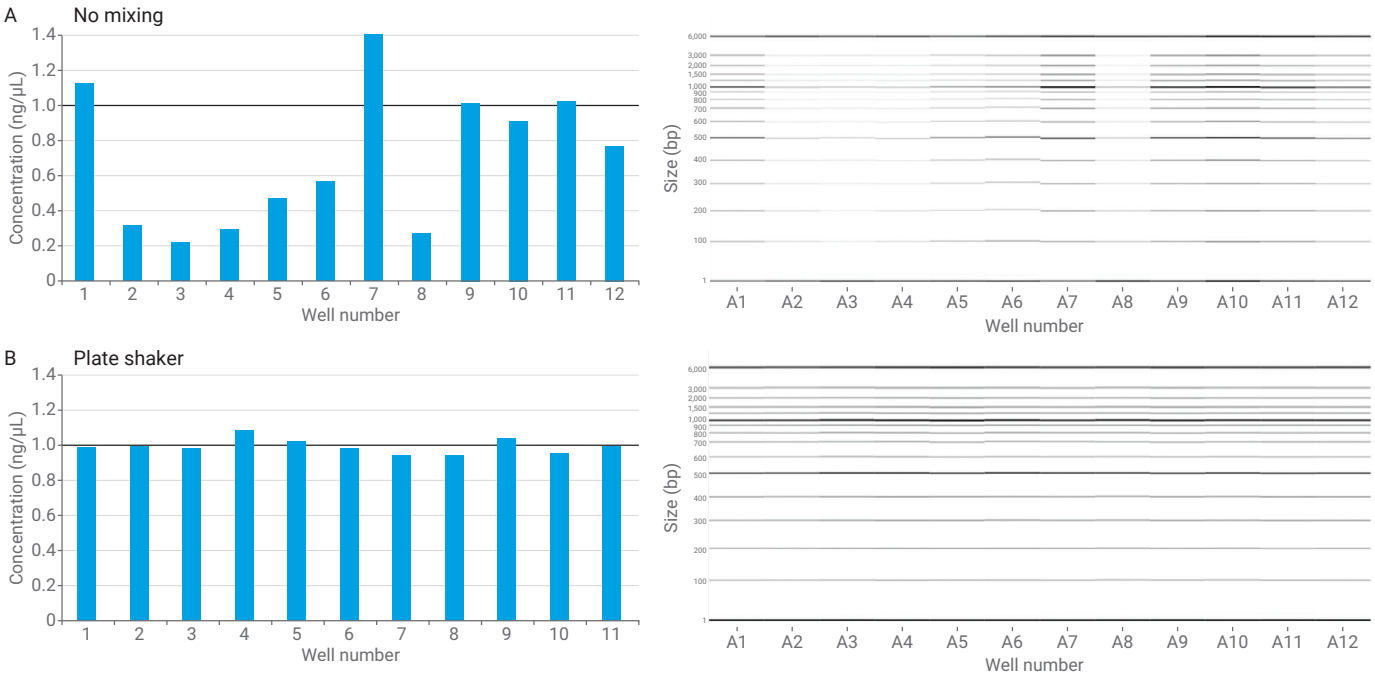
**Abstract:** QC of nucleic acids is essential for many downstream applications, including NGS. The Fragment Analyzer systems provide accurate and reliable sizing analysis of DNA smears and fragments with many reagent kits suited for different sizing ranges. This application note discusses the difference between peak size and average smear size provided by the ProSize data analysis software, how the presence of salt in your sample can affect analysis, and highlights the sizing accuracy and precision provided by the Fragment Analyzer systems.

Serial dilutions of two fragment sizes, 300 and 1,000 bp, were analyzed using the HS NGS Fragment kit (1–6000 bp). Serial dilutions of both fragments indicate that sizing of DNA fragments under 6,000 bp are not affected by concentration. An electropherogram overlay shows the consistent sizing of the 300 bp fragment at 15.6, 62.5, and 500 pg/µL (A). Similarly, a serial dilution of a DNA smear was analyzed using the HS Small Fragment kit, and the average smear size remained consistent over all concentrations (B). The Fragment Analyzer systems provide reliable sizing analysis for DNA fragments and smears for many sizes.

**Application note: 5994-0585EN**

# Analysis of DNA

## Best quantification practices



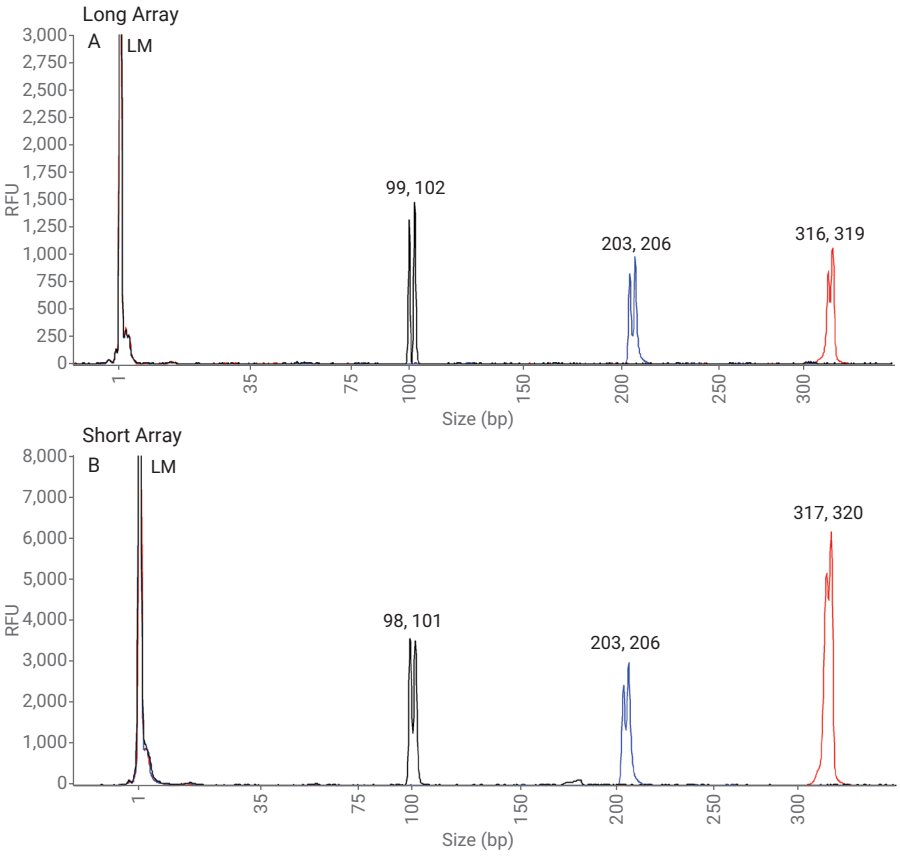

**Kits:** **HS NGS Fragment kit (1-6000 bp); Genomic DNA kit**

**Abstract:** Accurate and reliable quantification of nucleic acids is essential for many downstream applications, including PCR and next-generation sequencing library preparation. The Fragment Analyzer systems provide reliable quantification for fragments and smears. This application note describes best practices for measurement and factors that can affect analysis of nucleic acids. These include choosing the appropriate method for the sample, using the correct mixing method for each kit, proper pipetting technique, and sample preparation.

To demonstrate the effect that mixing can have on sample quantification, replicates of a ladder were prepared for analysis with the HS NGS Fragment kit. In the first run, the ladders were pipetted into the diluent marker and analyzed without any further mixing (A). For the second run, the samples and diluent marker were mixed together by plate shaker at 3,000 rpm for 2 minutes before analysis (B). The non-mixed samples exhibited a large variation in sample concentration, while the mixed samples showed a consistent quantification across all replicates. Following best practices for sample preparation will help ensure reliable and accurate DNA quantification with the Fragment Analyzer systems.

# Analysis of DNA

## Separation resolution of fragments



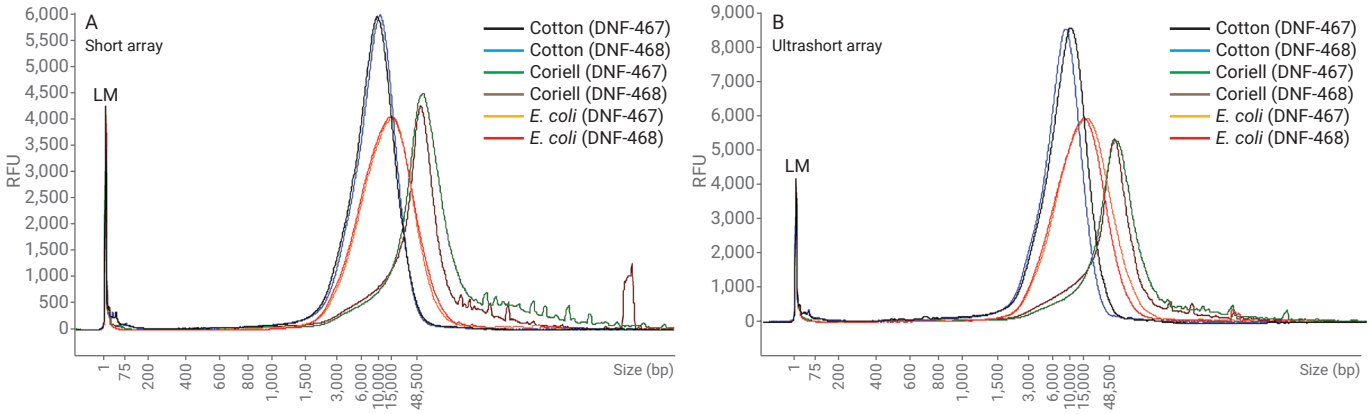

**Kits:** **dsDNA 905 Reagent kit (1-500 bp); HS Small Fragment kit**

**Abstract:** The ability to distinguish between two closely sized fragments is referred to as separation resolution in electrophoresis systems. Resolution of fragments is critical for accurate DNA sizing and identification of extra fragments when studying small PCR products. Traditional agarose gel-based systems lack the ability to resolve fragments close in size. The Fragment Analyzer system utilizes a unique gel chemistry, which allows for separation of closely sized DNA fragments, providing better insight into nucleic acid sample size. The HS Small Fragment kit and the dsDNA 905 Reagent kit (1-500 bp) are ideal for separating and sizing small PCR products. The electropherograms are overlays of the fragment mixes. Separation with the long array (A) produced a complete baseline resolution of the 101/104 bp fragments while displaying two partially separated peaks for the 201/204 and 306/309 bp fragment mixes. The short array (B) demonstrated a 3 bp separation as two partially resolved peaks for all three fragment mixes. The 5200 Fragment Analyzer system offers exceptional separation of similarly sized fragments with excellent precision and accuracy.

**Application note: 5994-0517EN**

# Analysis of DNA

## Consistent sizing of genomic DNA



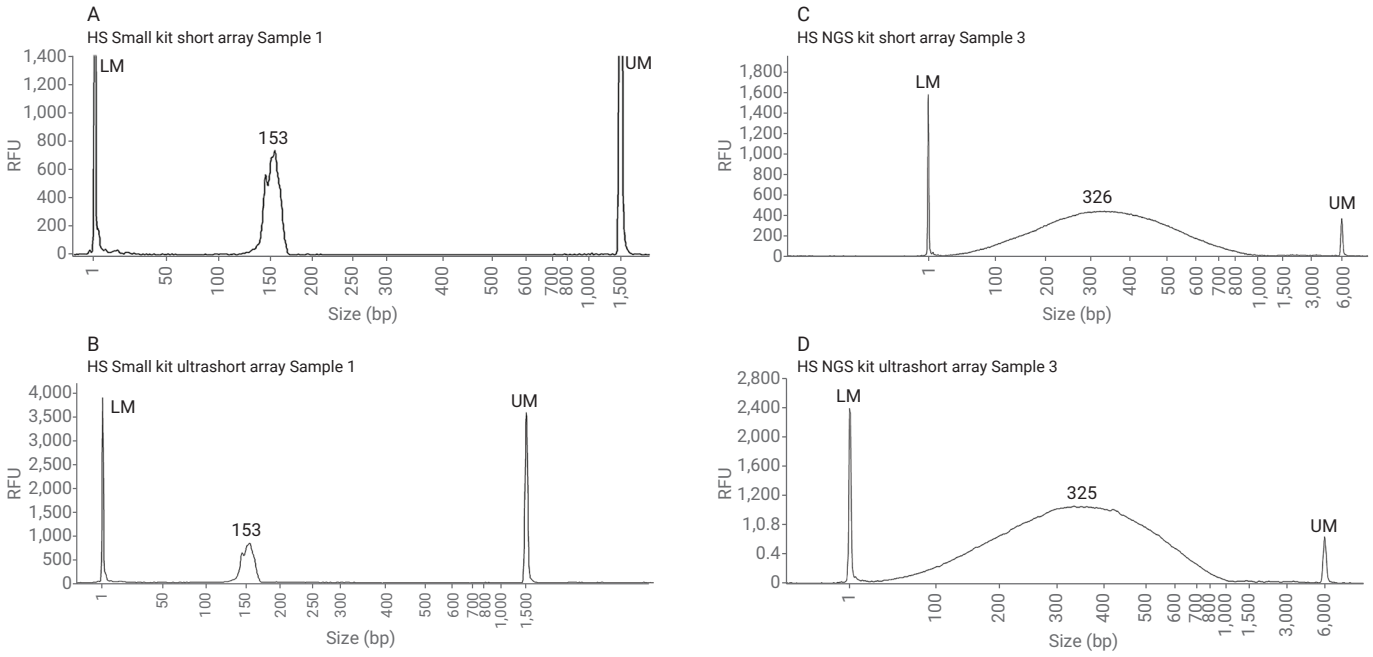

**Kits:** **Genomic DNA 50 kb kit; HS Genomic DNA 50 kb kit**

**Abstract:** The quality and concentration of gDNA starting material is crucial for successful downstream long-read and whole genome NGS. Quality analysis for gDNA with varying ranges of concentrations can be performed on the Fragment Analyzer systems with the Genomic DNA 50 kb kit (DNF-467) and the HS Genomic DNA 50 kb kit (DNF-468). The Genomic DNA 50 kb kit offers a concentration range of 25 to 250 ng/µL input gDNA, while the HS Genomic DNA 50 kb kit has a lower concentration range of 0.3 to 12 ng/µL input gDNA for low-concentrated samples. Genomic DNA from cotton, *E. coli*, and human (Coriell) were compared on both kits with the FA 12-Capillary Array Short, 33 cm (short array (A)) and FA 12-Capillary Array Ultrashort, 22 cm (ultrashort array (B)). On both kits, the short and ultrashort arrays demonstrated consistent sizing for the three sample types. The ultrashort array offers the convenience of shortened run times while providing comparable gDNA sizing, concentration, and GQN compared to the short array with both kits.

**Application note: 5994-0511EN**

# Next-Generation Sequencing

## Library size and quantification comparison with two kits



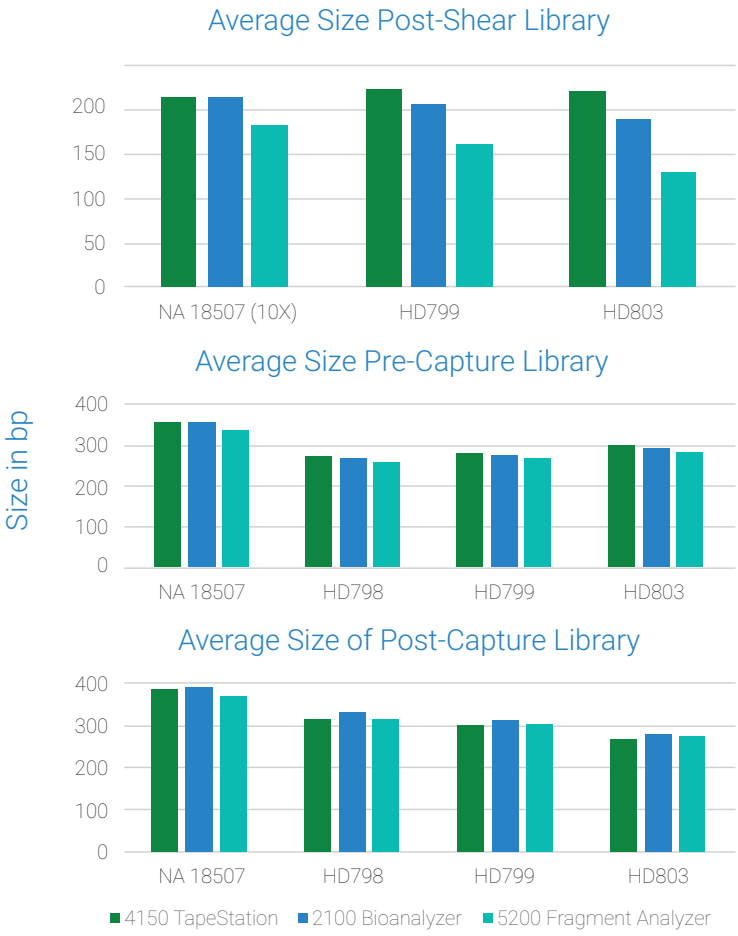

**Kits:** **HS Small Fragment kit; HS NGS Fragment kit (1-6000 bp)**

**Abstract:** The quality of NGS libraries is crucial to successful sequencing results. The Fragment Analyzer system offers easy analysis of sheared gDNA smears and libraries with the HS NGS Fragment kit (1-6000 bp) and the HS Small Fragment kit. The HS NGS Fragment kit sizes larger smears and fragments up to 6,000 bp, while the HS Small Fragment kit focuses on smaller sizes up to 1,500 bp. The FA 12-Capillary Array Ultrashort, 22 cm, decreases run time by 10 to 20 minutes compared to the standard FA 12-Capillary Array Short, 33 cm. The size and concentration of several DNA smears were compared between both kits and the short and ultrashort arrays. Library sizing and quantification remained consistent between the short and ultrashort arrays and both kits. The HS Small Fragment kit and the HS NGS Fragment kit can be used interchangeably for sizing and quantification of NGS libraries as long as the sample fits within the sizing specifications of the kit.

**Application note: 5994-0515EN**

# Next-Generation Sequencing

## Quality control in the Agilent Magnis SureSelect XT HS workflow




**Instruments:** **Magnis NGS Prep system; Fragment Analyzer systems**
**Kits:** **HS NGS Fragment kit (1-6000 bp)**

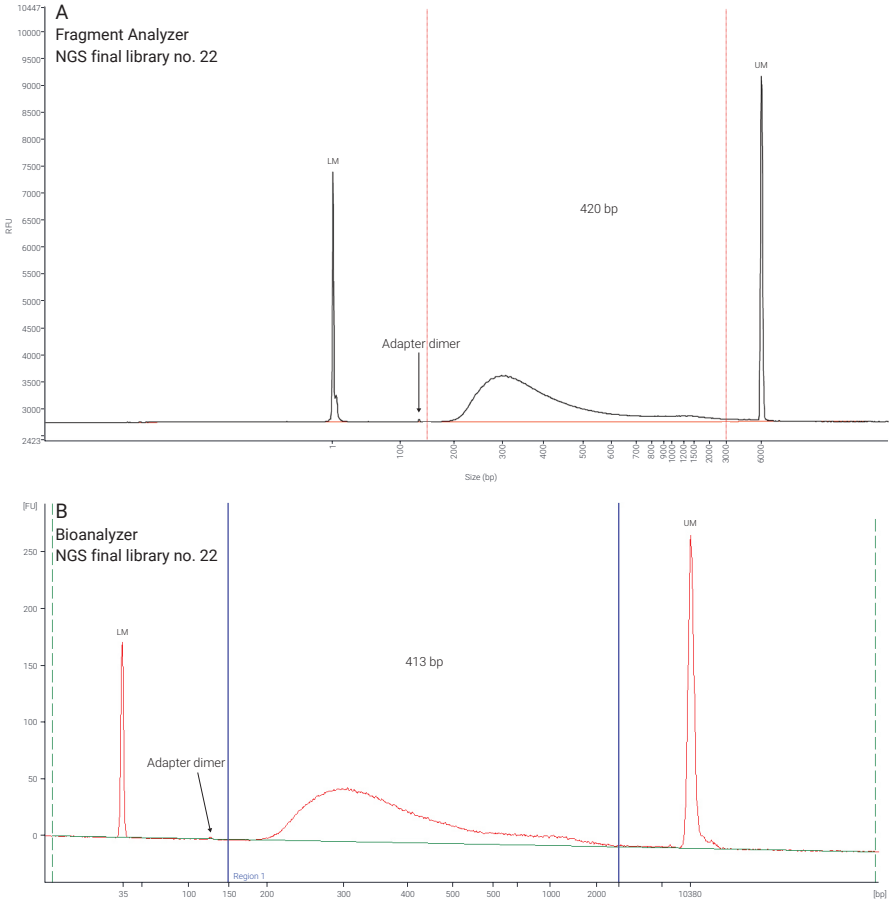
**Abstract:** The Agilent Magnis NGS prep system is an automated NGS library preparation solution for the Agilent SureSelect XT HS kit. It addresses challenges of manual library preparation such as hands-on time, expertise, optimization, and validation for diverse applications. It is beneficial to perform QC steps and quantification on the starting material, after fragmentation, the materials derived from intermediate steps (optional), and the final library to ensure reliability and overall success of the sequencing data. QC steps can be performed with the automated electrophoresis portfolio of instruments, including the Fragment Analyzer systems, Bioanalyzer system, and TapeStation systems.

Magnis SureSelect XT HS post-shear, pre-capture, and post-capture libraries were assessed on all three platforms and displayed similar sizing across multiple samples, as shown above. Each of the automated electrophoresis instruments provide reliable QC analysis, which is critical to ensure successful library preparation and sequencing.

**Application Note: 5994-1741EN**

# Next-Generation Sequencing

## Library comparison between the Fragment Analyzer and Bioanalyzer systems




**Instruments:** **Fragment Analyzer and Bioanalyzer systems**

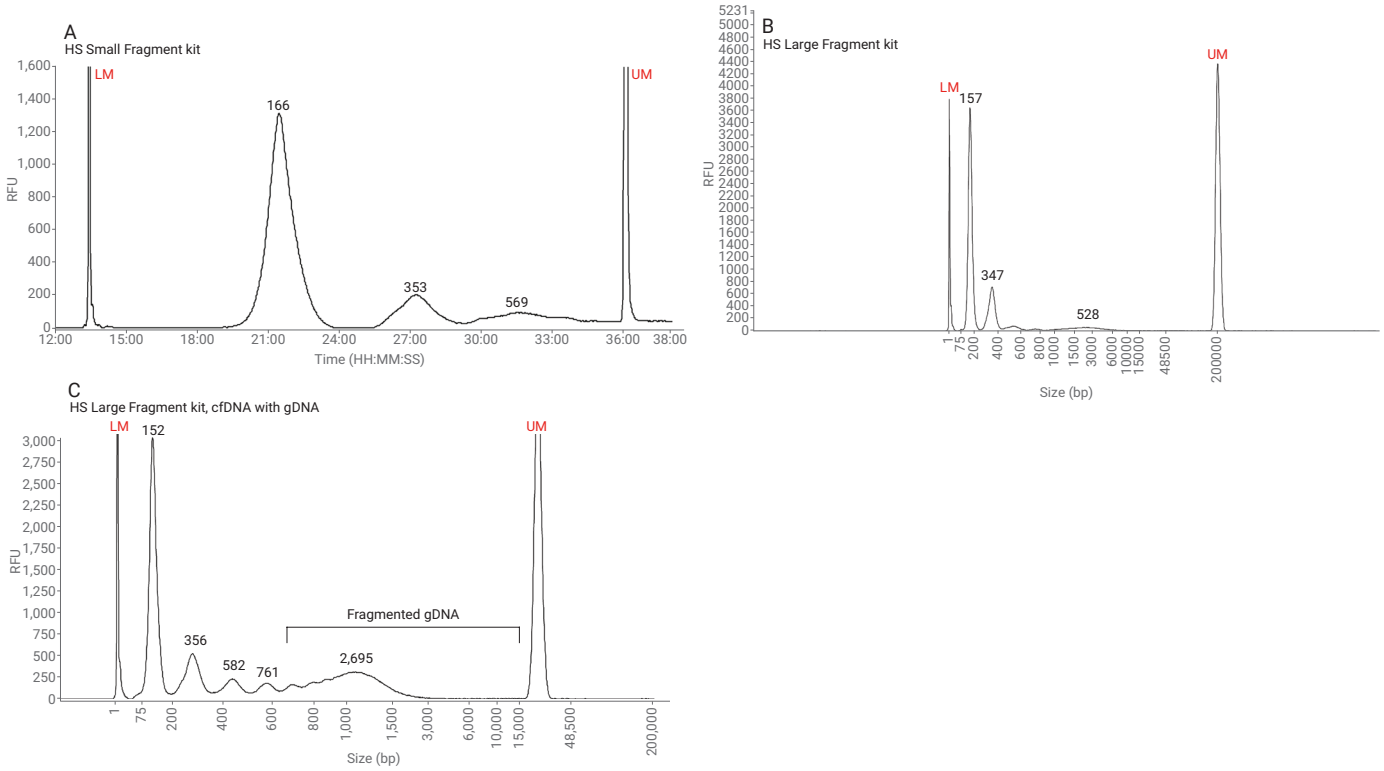
**Kits:** **Small Fragment and HS NGS Fragment kit (1-6000 bp) (Fragment Analyzer); DNA 1000 and HS DNA kit (Bioanalyzer)**

**Abstract:** Quality control throughout the NGS library preparation workflow is critical to ensure successful downstream sequencing. The Agilent Fragment Analyzer and Bioanalyzer systems provide reliable QC analysis for monitoring size, quantity, and molarity of the sample at different steps in the NGS workflow for two different library kits. The Small Fragment kit of the Fragment Analyzer system and the DNA 1000 kit of the Bioanalyzer system reported similar sizing, quantification, and molarity of NGS pre-capture libraries. In addition, the HS NGS Fragment kit of the Fragment Analyzer system (A) and the HS DNA kit of the Bioanalyzer system (B) displayed similar traces and reported similar sizing, quantification, and molarity for NGS final libraries. The high sensitivity of both instruments enabled them to detect a minute amount of adapter dimer contamination after cleanup with SPRI beads. Reliable and comparable QC results can be obtained from either the Fragment Analyzer system or the Bioanalyzer system, providing ease of mind when gathering data between two labs or transferring QC protocols from one system to the other.

**Technical overview: 5994-2459EN**

# Analysis of Cell-free DNA (cfDNA)

## Quality control analysis of cfDNA



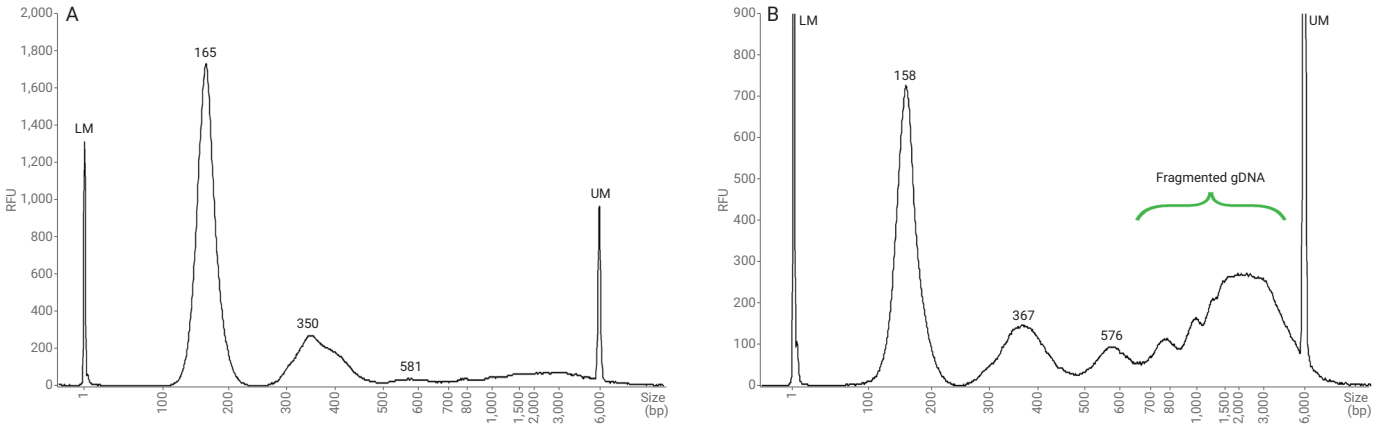

**Kits:** **HS Small Fragment kit; HS Large Fragment kit**

**Abstract:** Circulating cell-free DNA (cfDNA) is gaining prevalence as a noninvasive, alternative approach for the detection of tumor mutations in cancer management and screening tests for fetal abnormalities from the mother's blood. cfDNA is known to circulate in healthy and pathological conditions and is present in plasma, serum, cerebral spinal fluid, and saliva. A typical cfDNA electropherogram displays one, two, or three nucleosomal fragments. The HS Small Fragment kit distinctively separated three cfDNA peaks from healthy human serum at 166, 353, and 569 bp (A). These peak sizes corresponded to a nucleosome guided fragmentation pattern of apoptotic cfDNA, oftentimes referred to as mononucleosome, dinucleosome, and trinucleosome cfDNA. The same sample was also analyzed with the HS Large Fragment kit and three peaks were separated at 157, 347, and 528 bp (B). The HS Large Fragment kit also allows for reliable quantification and sizing for cfDNA samples containing fragmented genomic DNA (C).

**Application note: 5994-0510EN**

# Analysis of Cell-free DNA (cfDNA)

## Separation of cfDNA with the HS NGS Fragment kit



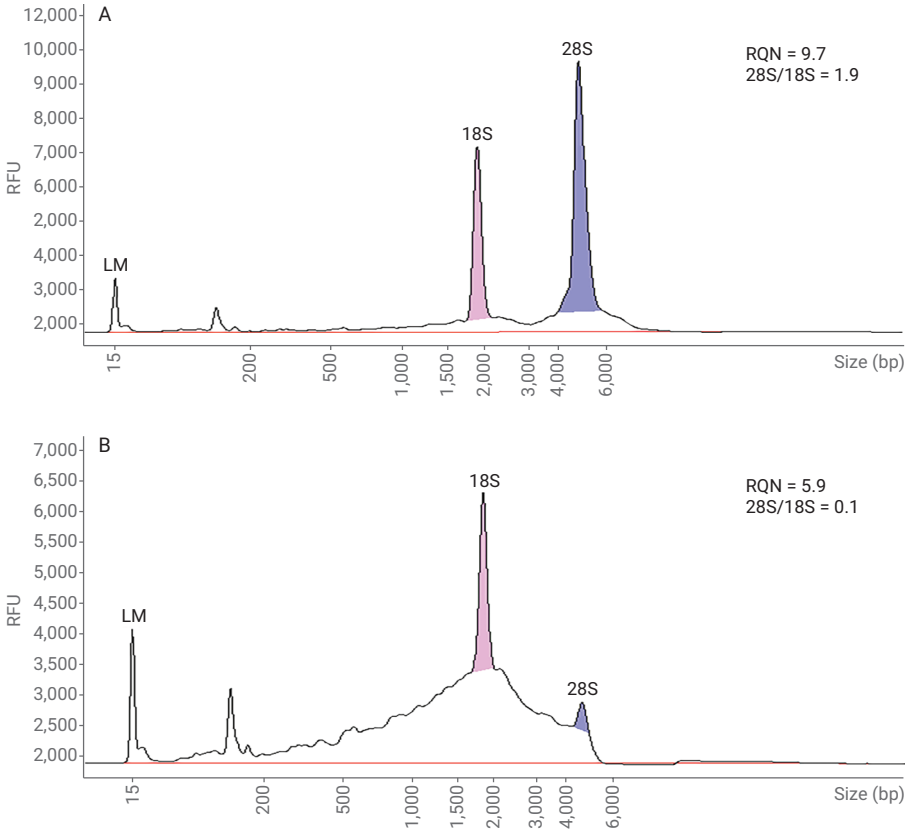

**Kits:** **HS NGS Fragment kit (1-6000 bp)**

**Abstract:** Quality analysis of extracted circulating cfDNA plays an important role in determining sizing and purity, thus providing knowledge that is necessary for sensitive downstream applications such as NGS. cfDNA was extracted from healthy human serum using the Quick-cfDNA serum and plasma kit from Zymo. Typical cfDNA separation profiles display two or three distinct peaks (A). Extraction of fragmented gDNA with cfDNA can occur. The results showed that the HS NGS Fragment kit (1-6000 bp) was able to effectively separate the three nucleosome cfDNA peaks from gDNA (B). Some extraction kits have the option to use carrier RNA during cfDNA extraction. Carrier RNA was shown to comigrate with the dinucleosome peak on the HS NGS Fragment kit, inflating the concentration of the second peak. It is recommended to take into consideration the cfDNA extraction methods when analyzing cfDNA.

**Application note: 5994-0522EN**

# Analysis of RNA

## RQN quality metrics



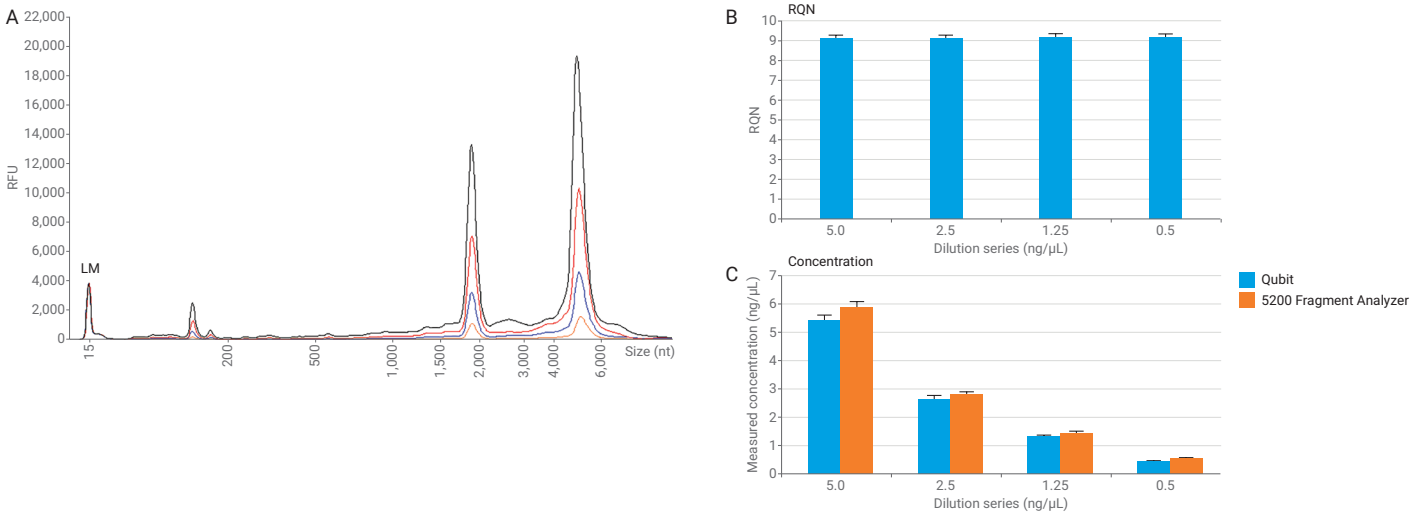

**Kits:** **HS RNA kit (15 nt)**

**Abstract:** The RQN is a user-independent quality metric for easy evaluation of total RNA quality. Total RNA quality is a constant concern because of how easily RNA degrades due to temperature, enzymatic degradation from the abundance of RNase in the environment, and improper handling. The Fragment Analyzer system and RQN metric were utilized to analyze universal mouse reference total RNA degradation. Samples treated for 0 minutes at 70 °C resulted in an RQN of 9.7 (A), while 10 minutes at 70 °C degraded the sample to an RQN of 5.9 (B). Electropherograms allow for the total RNA profiles to be compared at different points of degradation. The concentration, RQN, and ribosomal ratio are automatically reported in ProSize, allowing for easy evaluation of total RNA quality.

**Application note: 5994-0521EN**

# Analysis of RNA

## Eukaryotic total RNA



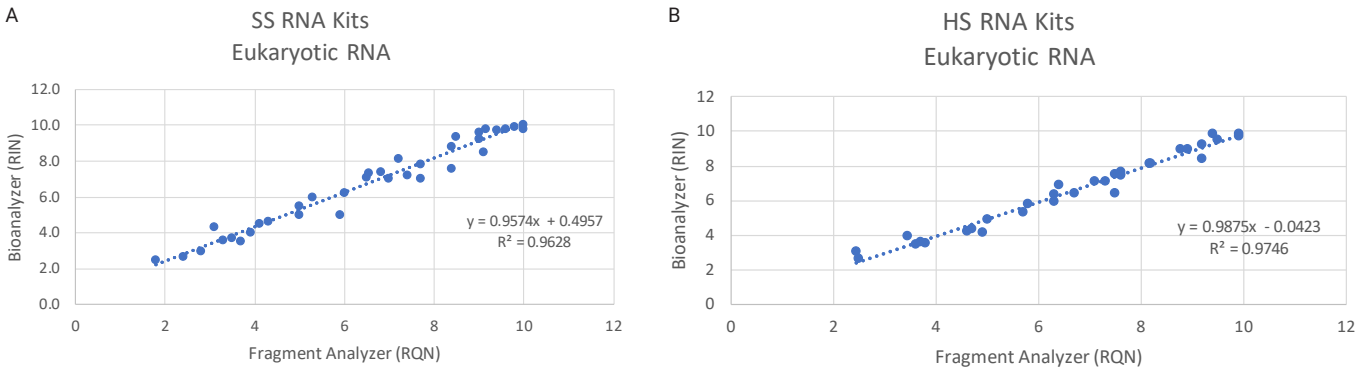

**Kits:** **HS RNA kit (15 nt)**

**Abstract:** High-quality total RNA is critical for successful outcomes in RT-PCR, microarray analysis, and NGS. The Fragment Analyzer systems independently assign an RQN that justly represents the sample quality. 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 HS RNA kit (15 nt) throughout the concentration range of the kit. Excellent separation resolution was seen between the 18S and 28S ribosomal peaks and in the small RNA region. As shown in the overlay (A), the sizing of the 18S and 28S ribosomal peaks remained constant and was unchanged by the sample concentration. The RQN also remained consistent across the dilution series, averaging 9.2 ± 0.1 (B). The concentration of each sample (approximately 5, 2.5, 1.25, and 0.5 ng/µL) was analyzed and compared between the Qubit and 5200 Fragment Analyzer systems (C). Both the Qubit and the Fragment Analyzer systems reported similar concentrations throughout the dilution series with a percent error of 8% or less.

**Application note: 5994-0519EN**

# Analysis of RNA

## Comparison of the RIN and RQN



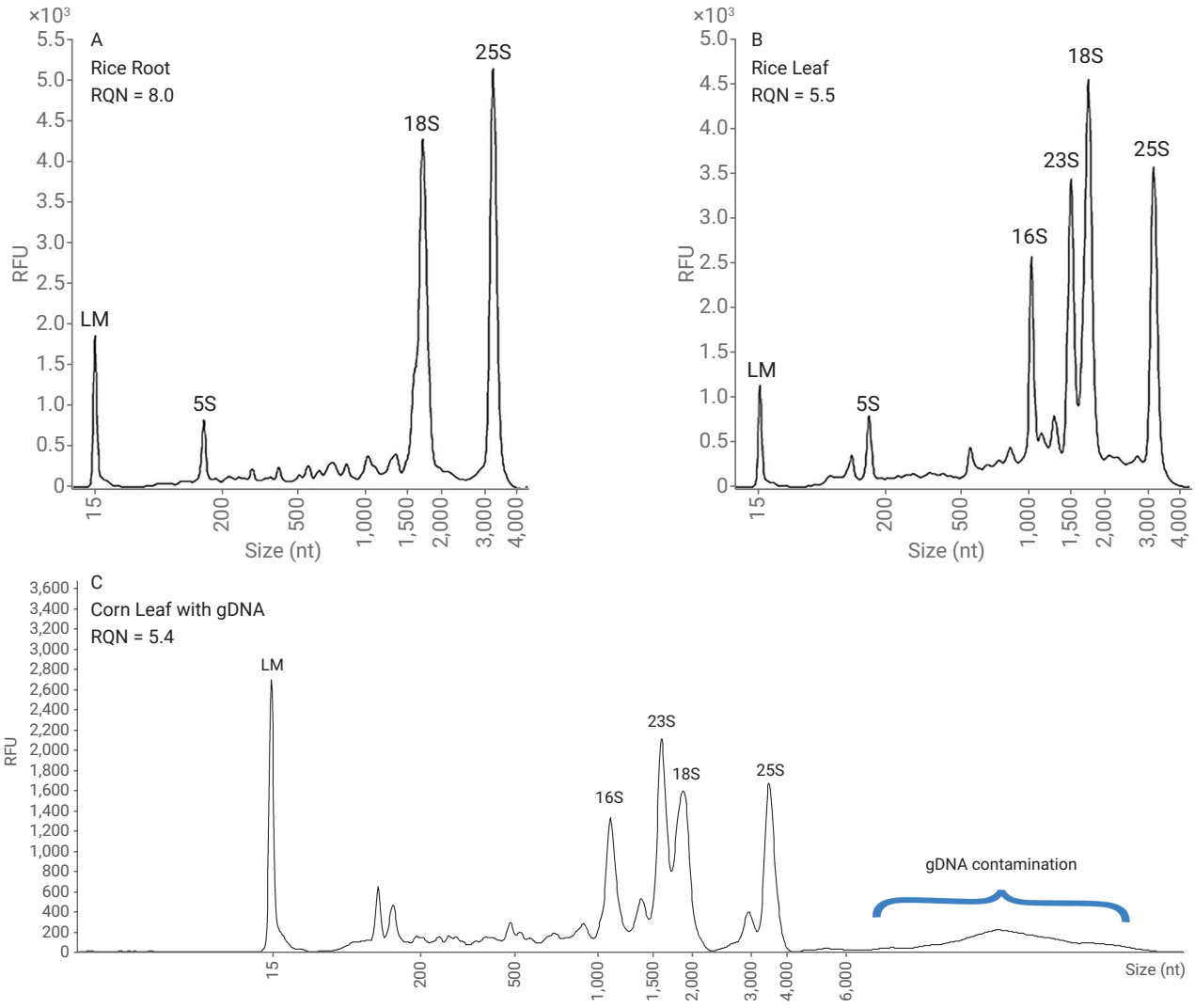

**Instruments:** **Fragment Analyzer and Bioanalyzer systems**

**Kits:** **RNA (15 nt) and HS RNA (15 nt) kits (Fragment Analyzer); RNA 6000 nano and RNA 6000 pico kits (Bioanalyzer)**

**Abstract:** The Bioanalyzer instrument is well established for providing a reliable, automated RNA integrity number (RIN). The RIN provides an objective assessment of RNA integrity. The Fragment Analyzer offers a user-independent quality metric, the RQN, for easy evaluation of total RNA quality. Both the RIN and RQN consider the entire electropherogram with scoring from 10 to 1, where 10 indicates the highest possible RNA quality and 1 completely degraded RNA. Eukaryotic samples with a varying degree of RNA integrity from completely intact, to mildly and strongly degraded were compared on the Bioanalyzer and Fragment Analyzer instruments. Both the standard-sensitivity (A) and high-sensitivity RNA (B) kits for both instruments provided comparable RIN and RQN scores throughout the degradation series. This is demonstrated with the slope and $R^2$ value close to 1. This technical overview also shows a strong correlation between the RIN and RQN for prokaryotic *E. coli* RNA samples.

# Analysis of RNA

## Assessing quality of plant RNA



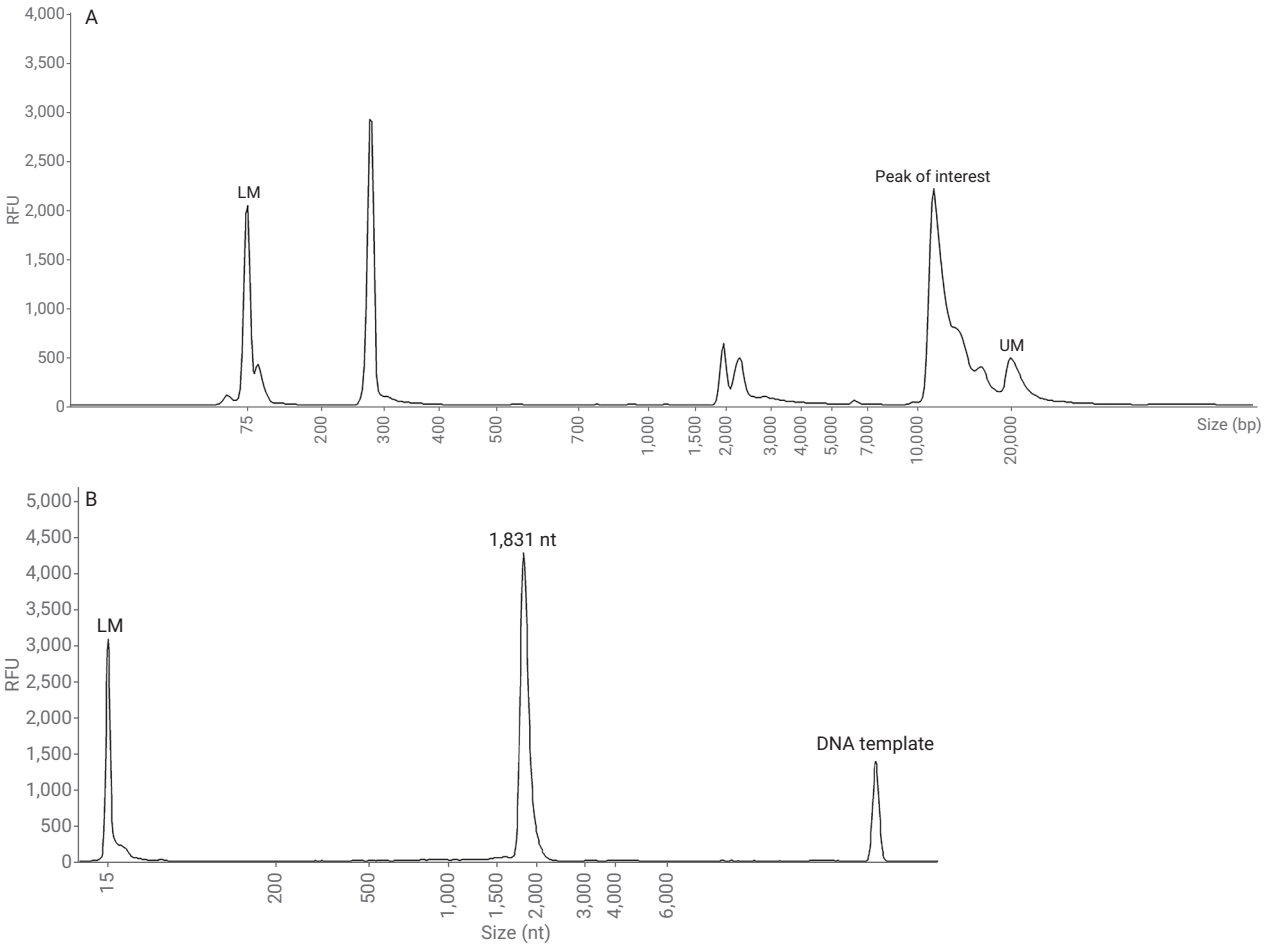

**Kits:** **HS RNA kit**

**Abstract:** Plant tissues have three types of ribosomal RNAs (rRNA): chloroplast, cytosolic, and mitochondrial. The 5200 Fragment Analyzer system delivers ample resolution to separate all four leaf rRNA peaks: 16S, 23S, 18S, and 25S. The ProSize data analysis software has a dedicated plant mode for evaluating complex plant RNA samples. For example, the electropherograms pictured highlight the difference between rice root (A) and leaf RNA (B). Leaf samples have additional chloroplast rRNA (16S and 23S) not present in root RNA. High-quality RNA, free of gDNA, is critical for the success of many downstream techniques including RT-PCR, microarray analysis, and NGS. The electropherogram displayed in ProSize software provides an easy visual in determining if any gDNA contamination is present in the RNA sample. Figure C shows an example of corn leaf RNA with gDNA contamination.

**Application note: 5994-0518EN**

# Analysis of IVT mRNA

## Quality control of IVT mRNA



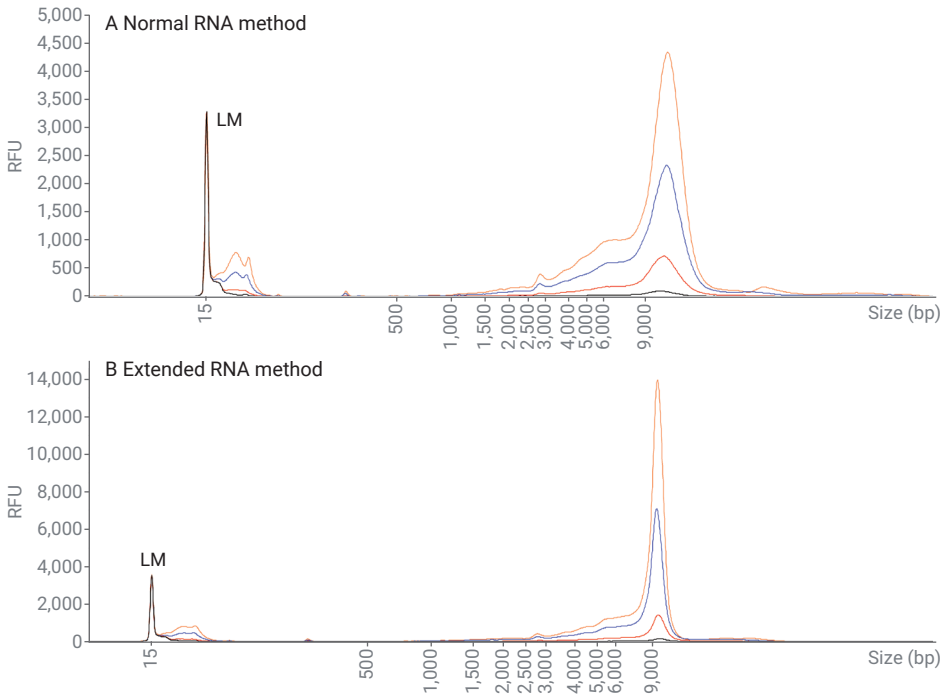

**Kits:** **RNA kit (15 nt); HS RNA kit (15 nt); dsDNA 930 Reagent kit (75-20000 bp)**

**Abstract:** QC analysis is an essential part of the *in vitro* transcription (IVT) RNA workflow. QC during IVT allows for detection of poor PCR amplification, poor transcription, RNA contamination from DNA template, and degraded RNA, as well as ensuring that downstream applications start with high-quality RNA. The first recommended QC checkpoint helps to determine if the PCR amplification and product cleanup procedures were successful. PCR product was analyzed on the Fragment Analyzer system with the dsDNA 930 Reagent kit (A). Multiple unwanted peaks indicated a poor PCR reaction. The second recommended QC checkpoint occurs after transcription. Cleanup of the transcription product involves the use of DNase to eliminate the DNA template, leaving a purified RNA product. The Fragment Analyzer systems can detect the presence of a DNA template in RNA (B). Detection of degraded, contaminated, or otherwise unsuitable RNA allows researchers to rework or remove these samples early in the process.

**Application note: 5994-0512EN**

# Analysis of IVT mRNA

## Long IVT mRNA sizing



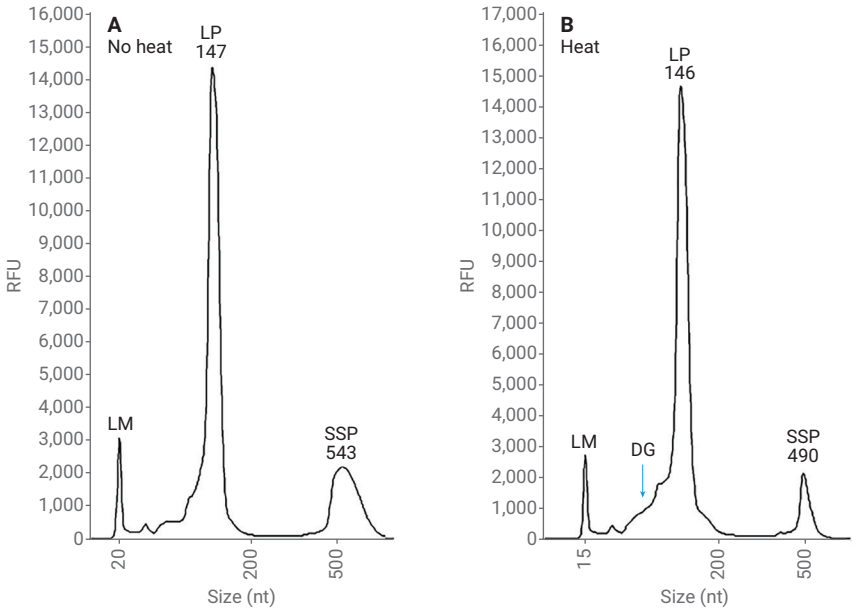

**Kits:** **RNA kit (15 nt)**

**Abstract:** The use of IVT mRNA is becoming widespread in research areas such as ribozyme and aptamer synthesis, mRNA synthesis, RNA interference, and antisense RNA techniques. Longer RNA transcripts greater than 3,000 nt are needed for gene structure and functional studies. Reliable sizing and quality assessment of IVT mRNA at 10,000 nt was obtained using the 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) with a normal (8 kV, 45 minutes) and an extended RNA separation method (4 kV, 90 minutes). A dilution series over the entire concentration range of the kit (100, 50, 10 and 1 ng/µL) of the 10,000 nt IVT mRNA sample was assessed. The average size throughout the dilution series for the normal (A) and extended method (B) respectively, was 10,526 and 9,543 nt with a low percent coefficient of variance (% CV) of 0.4% and 0.5% and a low percent error of 5.2% and -4.6%, indicating precise and accurate sizing for both methods. The extended method provided sharper peaks enabling the ability to distinguish minute amounts of degradation.

**Application note: 5994-0878EN**

# CRISPR/Cas Engineering

## Single-guide RNA quality assessment



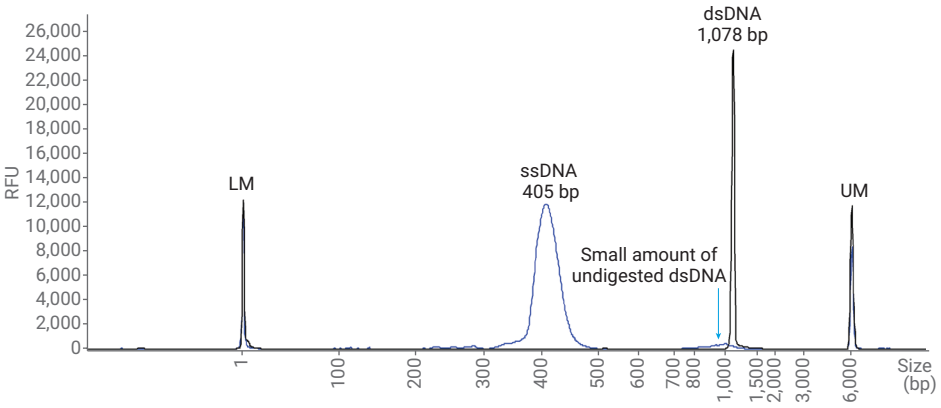

**Kits:** **HS RNA kit (15 nt)**

**Abstract:** One method of CRISPR/Cas9 gene editing is the delivery of Cas9 complexed *in vitro* with a single-guide RNA (sgRNA). Traditionally, quality control analysis of sgRNA prior to Cas9 complexing is performed by agarose gel electrophoresis. While this process can determine whether an sgRNA is extensively degraded, the resolution and sensitivity of agarose gels does not allow for detection of low levels of degradation that can affect editing efficiency. Heat denaturing RNA before separation by electrophoresis reduces extensive secondary structure peaks (SSP) formed by intramolecular base pairing, which may inhibit size-dependent migration of RNA fragments. Separation of sgRNAs with (B) and without (A) heat denaturation was performed on the 5200 Fragment Analyzer system with the HS RNA kit. sgRNAs are capable of folding into many different secondary structures. The presence of denaturing formamide in the diluent marker of the kit was adequate to limit sgRNA secondary structure formation without heat denaturation in most samples.

**Application note: 5994-0523EN**

# CRISPR/Cas Engineering

## Analysis of long, single-stranded DNA



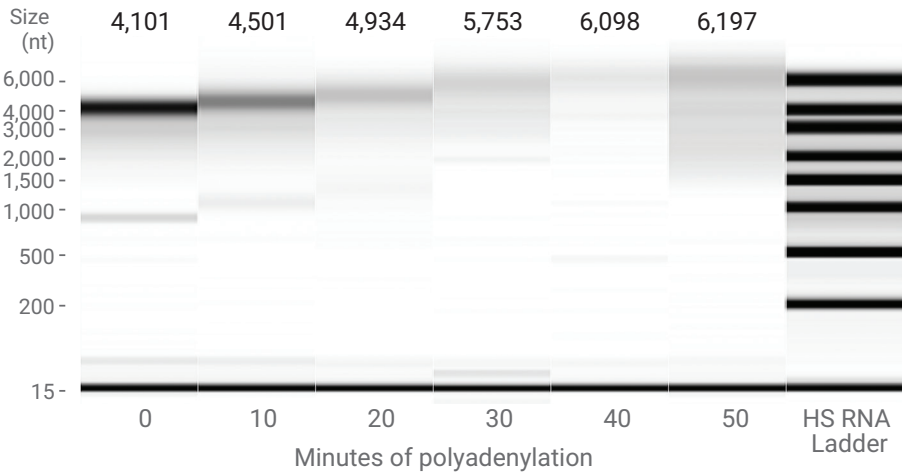

**Kits:** **CRISPR Discovery Gel kit**

**Abstract:** Single-stranded DNA (ssDNA) templates are becoming increasingly useful in CRISPR/Cas homology-directed repair (HDR). ssDNA templates, ranging from 500 base to kilobase length, have been found to greatly increase the number of homologous recombination events when compared to the traditional double-stranded DNA (dsDNA) templates. Creation of ssDNA templates requires quality control steps to ensure complete conversion from dsDNA to ssDNA. Analysis of ssDNA prepared from digestion of dsDNA was analyzed on the 5200 Fragment Analyzer system. The overlay of ssDNA digest product (blue) and beginning dsDNA (black) shows a minute amount of undigested dsDNA left over after digestion. The high sensitivity and resolution of the Fragment Analyzer instrument allowed for detection of small amounts of undigested dsDNA not detectable on agarose gel.

**Application note: 5994-0584EN**

# CRISPR/Cas Engineering

## Polyadenylation of Cas9 mRNA



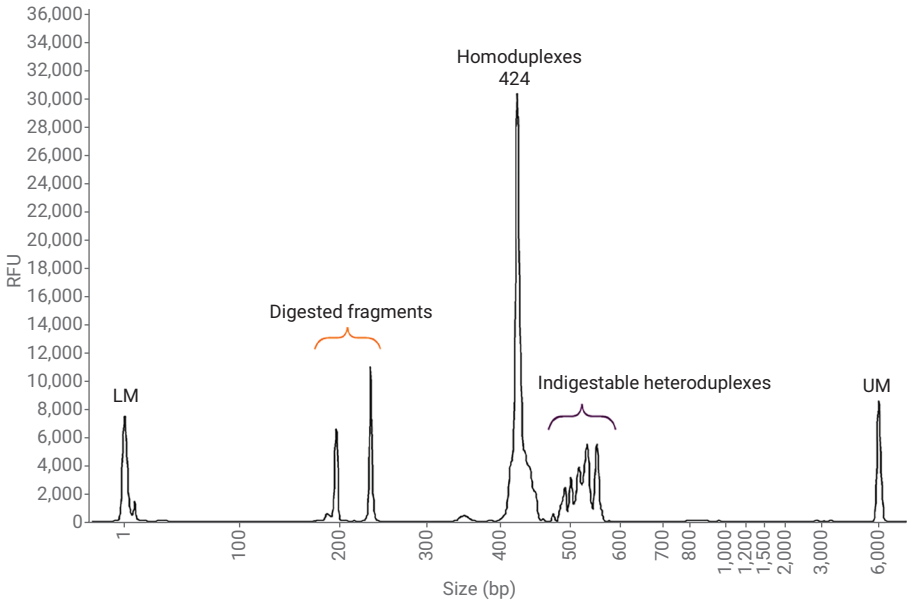

**Kits:** **HS RNA kit (15 nt)**

**Abstract:** DNA-free CRISPR gene editing has become a popular way to control for off-target effects during CRISPR transfection. However, the size of the Cas9 protein does not always allow for transfection of Cas9/sgRNA ribonucleoprotein complexes. A way to overcome this method of DNA-free CRISPR gene editing is to transfect both *in vitro*-transcribed sgRNA and Cas9 mRNA. Polyadenylation of the Cas9 mRNA allows for a longer translation time by protecting and increasing the stability of the Cas9 transcript. Above is a digital gel image of Cas9 mRNA with polyadenylation reaction time = 0, 10, 20, 30, 40, and 50 minutes separated on the 5200 Fragment Analyzer system with the HS RNA kit (15 nt). The size increases with increased polyadenylation. The Fragment Analyzer instrument provides high-resolution separation and consistent sizing of the Cas9 mRNA with or without polyadenylation.

**Application note: 5994-0583EN**

# CRISPR/Cas Engineering

## Engineered restriction site to estimate CRISPR homology-directed repair efficiency




**Kits:** **CRISPR Discovery Gel kit**

**Abstract:** The 5200 Fragment Analyzer system coupled with the CRISPR Discovery Gel kit provides high-throughput analysis for detection of CRISPR-induced, homology-directed repair (HDR) knock-in of novel restriction enzyme sites. The presented method enables determination of the approximate frequency of HDR repairs in both pooled and individual cell line systems when using CRISPR gene editing. Analysis of BamHI digested from 40% pHDR mixed template PCR using the 5200 Fragment Analyzer system with the CRISPR Discovery Gel kit. Heteroduplex formation was easily seen on the 5200 Fragment Analyzer system, providing for rapid assessment of cleavage accuracy.

**Application note: 5994-0524EN**

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