Technical Overview

Agilent
Trusted Answers

Analysis of Total RNA on the Agilent Femto Pulse System with the Agilent Ultra Sensitivity RNA Kit

Introduction

The Agilent Femto Pulse system is a powerful and effective pulsed-field capillary electrophoresis system used for quantification, sizing and quality control of nucleic acid samples. The Femto Pulse was designed with low concentration samples and high molecular weight (HMW) DNA in mind. The optimized optical platform allows the system to easily achieve higher sensitivity for nucleic acid smear and fragment analysis, compared to conventional gel electrophoresis[1]. The unparalleled sensitivity requires minimal precious sample and achieves superior detection with the ability to quantify a single cell's worth of genomic DNA or total RNA. The Femto Pulse is also capable of pulsed-field separation of HMW genomic DNA, providing a more detailed electropherogram representation and accurate size of the genomic DNA. The system is therefore ideal for quality control (QC) analysis of samples for downstream applications, including single-cell analysis, cfDNA[2], PCR-free libraries, RNA, genomic DNA[3], bacterial artificial chromosomes (BAC)[4,5], and long-read sequencing libraries[6].

The Agilent Ultra Sensitivity RNA kit (US RNA kit) can be used for assessment of both total RNA and mRNA from 200–6,000 nt at low concentrations. There are two optimized methods that allow for detection down to 2.5 pg/µL total RNA or 15 pg/µL mRNA, and quantification up to 250 pg/µL total RNA or 500 pg/µL mRNA. The US RNA kit can be used for analysis of total RNA, mRNA, and *in vitro* transcribed (IVT) mRNA fragments, providing reliable concentration and sizing. The powerful Agilent ProSize data analysis software provides an objective quality score - the RNA quality number (RQN)[7] - in addition to concentration, fragment sizing, and a calculation of the percent ribosomal contamination for mRNA.

Ensuring the quality of RNA is crucial to many downstream applications, including RNA sequencing, NGS and gene expression studies. Conventionally, the ribosomal peak ratio of 28S/18S was used to assess the quality of RNA. The Femto Pulse system utilizes the RQN as a quality metric indicator for fresh or frozen total RNA. The RQN takes the entire electropherogram into consideration, including the 5S and fast region where the small RNA separates, as well as the small (18S) and large (28S) ribosomal peaks, the baseline resolution between the ribosomal 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 based on a scale from 1 to 10, where a high RQN indicates highly intact RNA, and a low RQN indicates a strongly degraded RNA sample. The ProSize software automatically reports the 28S/18S ratio, RQN, and concentration of the RNA sample. Excellent resolution allows for distinction between small RNA and degraded RNA, providing a reliable and accurate RQN score.

In this technical overview, the reproducibility of the Femto Pulse system is presented throughout the concentration range and over a degradation series of the US RNA kit. In addition, the consistency of quantification and RQN quality metric score is analyzed between two Femto Pulse systems. Both the Femto Pulse and the Agilent Fragment Analyzer are capillary electrophoresis instruments that utilize the RQN quality metric. The Femto Pulse system is ideal for analysis of samples with low concentration and for pulsed-field separation of gDNA, while the Fragment Analyzer system analyzes RNA samples that are three times higher in concentration and accommodates low to high throughput flexibility of 12–96 samples at one time (Table 1). In order to demonstrate that the RQN is analogous between the two systems, the RQN correlation was examined over a wide range of RNA degradation.

Table 1. Specification table of the RNA kits for the Agilent Fragment Analyzer and Femto Pulse systems.

RNA Kit Specifications		
Instrument	**RNA kit**	**Concentration range**
Femto Pulse	Ultra Sensitivity RNA kit	15-250 pg/µL
Fragment Analyzer	RNA kit (15 nt)	25-500 ng/µL
	High Sensitivity RNA kit (15 nt)	Total RNA 50-5,000 pg/µL mRNA 500-5,000 pg/µL

Experimental

Total RNA was diluted from 250 to 16 pg/µL with the Agilent FP RNA Dilution Buffer (p/n FP-6501-0003) included in the Ultra Sensitivity RNA kit (p/n FP-1201-0275) and analyzed on the Femto Pulse system (p/n M5330AA). For the degradation studies and instrument comparison between the Agilent 5200 Fragment Analyzer (p/n M5310AA) and Femto Pulse systems, different total RNA samples from human, rat and mouse were obtained from commercial vendors Zyagen and Agilent Technologies. Samples were diluted with RNase-free water to 400 ng/µL and heat-degraded with the Agilent SureCycler 8800 thermal cycler (p/n G880A) for 0 to 20 minutes at 90 °C in order to generate a variety of degraded RNA samples, ranging from completely intact, to mildly and strongly degraded. The degraded samples analyzed on the Femto Pulse system were further diluted with the FP RNA Dilution Buffer. Both the Agilent RNA kit (15 nt) (p/n DNF-471) and the HS RNA kit (15 nt) (p/n DNF-472) were utilized on the Fragment Analyzer system.

Results and discussion

RNA dilution series

Mouse total RNA was diluted throughout the concentration range of the US RNA kit and analyzed on the Femto Pulse system. The electropherogram overlay demonstrates uniformity of separation and alignment of the 18S and 28S peaks throughout the dilution series (Figure 1A). The RQN provided by the ProSize software remained consistent throughout the concentration range of the kit (Figure 1B). The Femo Pulse provides reliable separation analysis and quality metric scores for picogram levels of total RNA.

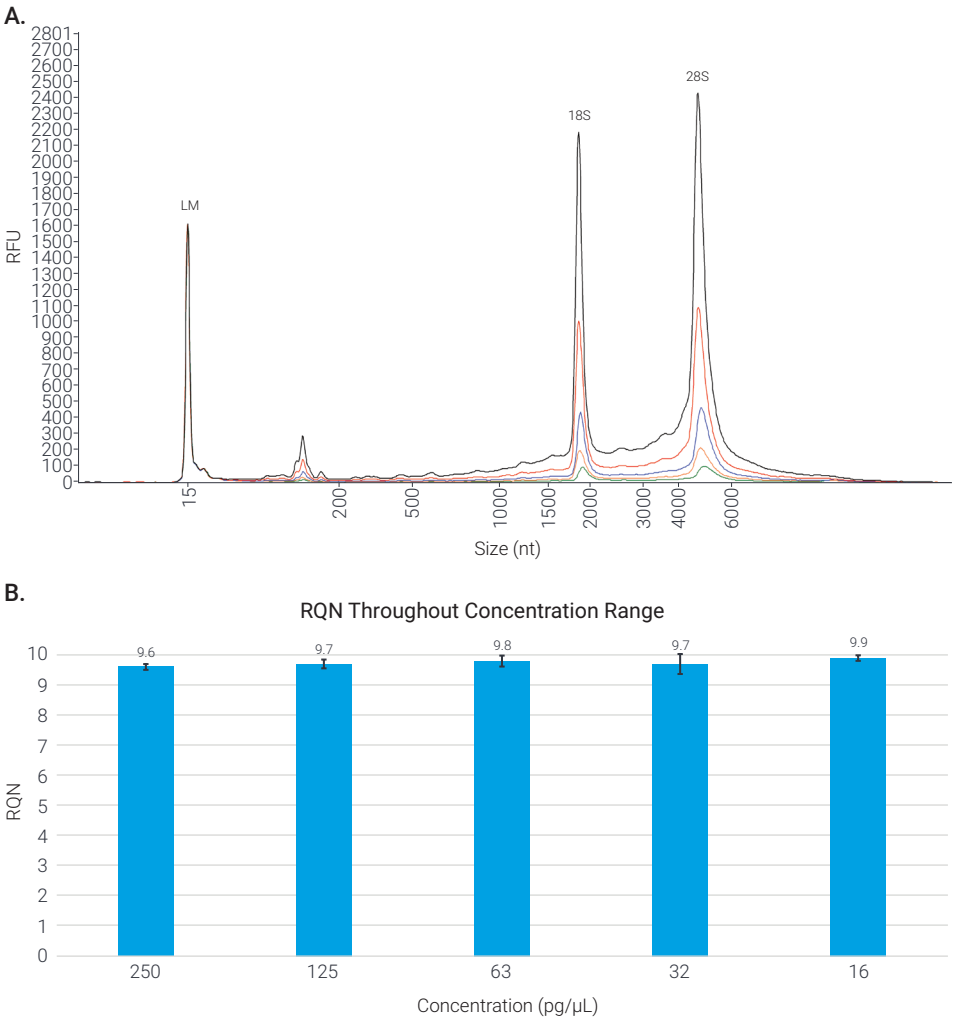
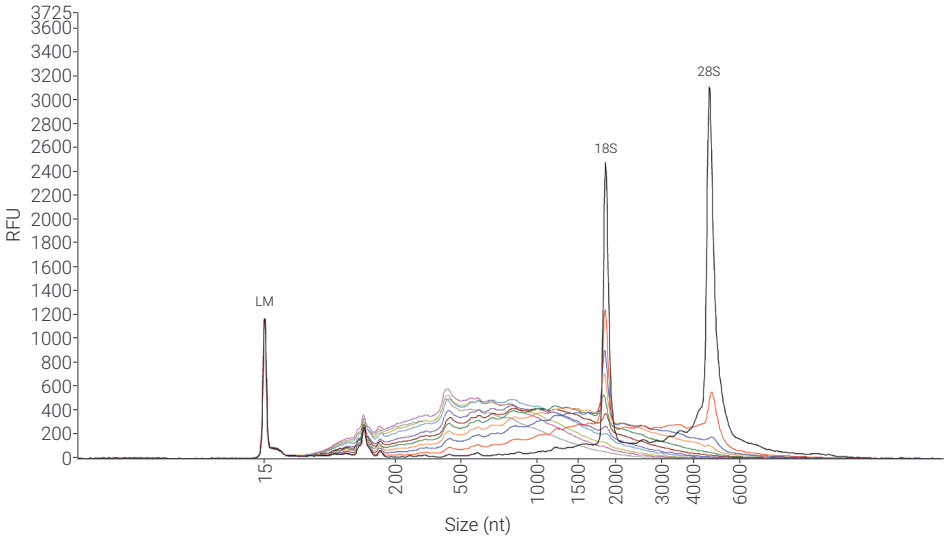


Figure 1. Mouse total RNA through the concentration range on the Agilent Ultra Sensitivity RNA kit and Femto Pulse system. (A) Electropherogram overlay. (B) RQN throughout the concentration range, n = 5. LM = lower marker.

RNA degradation series

RNA is easily degraded through handling and environmental factors, including heat and RNases. Thus, it is important to assess RNA quality prior to working with it. The RQN quality metric score was assessed with degradation of mouse total RNA by heat denaturation at 90 °C for varying amounts of time. Analysis with the Femto Pulse system revealed the rapid degradation of the 28S ribosomal peak in the electropherogram (Figure 2A) and digital gel image (Figure 2B). As expected, the RQN quality metric score steadily decreased in correlation with the loss of the 28S peak, decrease of 18S, and continued degradation.

A. Mouse Total RNA degradation series



B. RQN Mouse Total RNA degradation series

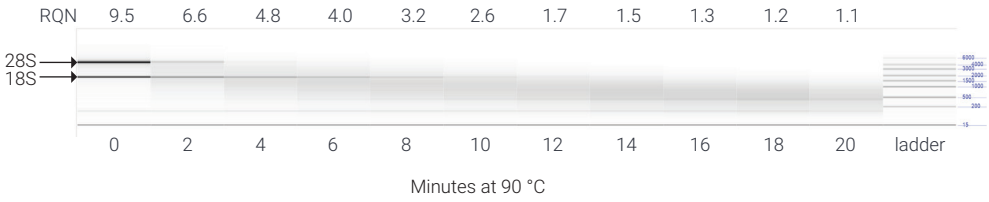


Figure 2. Degradation series of mouse total RNA heat denatured at 90 °C for varying amounts of time with the Agilent Ultra Sensitivity RNA kit on the Femto Pulse system. (A) Electropherogram overlay. (B) Digital gel image. LM = lower marker.

Instrument comparison

The reproducibility between Femto Pulse instruments was analyzed for RNA concentration and quality. The dilution series of mouse total RNA was compared between two Femto Pulse systems. Both instruments reported similar concentrations throughout the dilution series (Figure 3A). The concentration precision (%CV) and accuracy (%error) on each instrument were below the quantification specifications of the US RNA kit. The RQN quality metric also remained consistent between the two Femto Pulse systems throughout the dilution series (Figure 3B) with a precision below 4% CV for all concentration points. The RQN was analyzed with mouse liver RNA throughout a degradation series and remained consistent between two Femto Pulse instruments (Figure 3C). Not all RNA will degrade at the same rate, resulting in different RQN quality scores. This can be seen in Figure 2B, with mouse total RNA degrading faster than mouse liver RNA (Figure 3C). Factors other than tissue type which can affect RNA quality include the amount of RNases in the tissue, volume, concentration and temperature. Therefore, it is important to verify RNA quality prior to working with it.

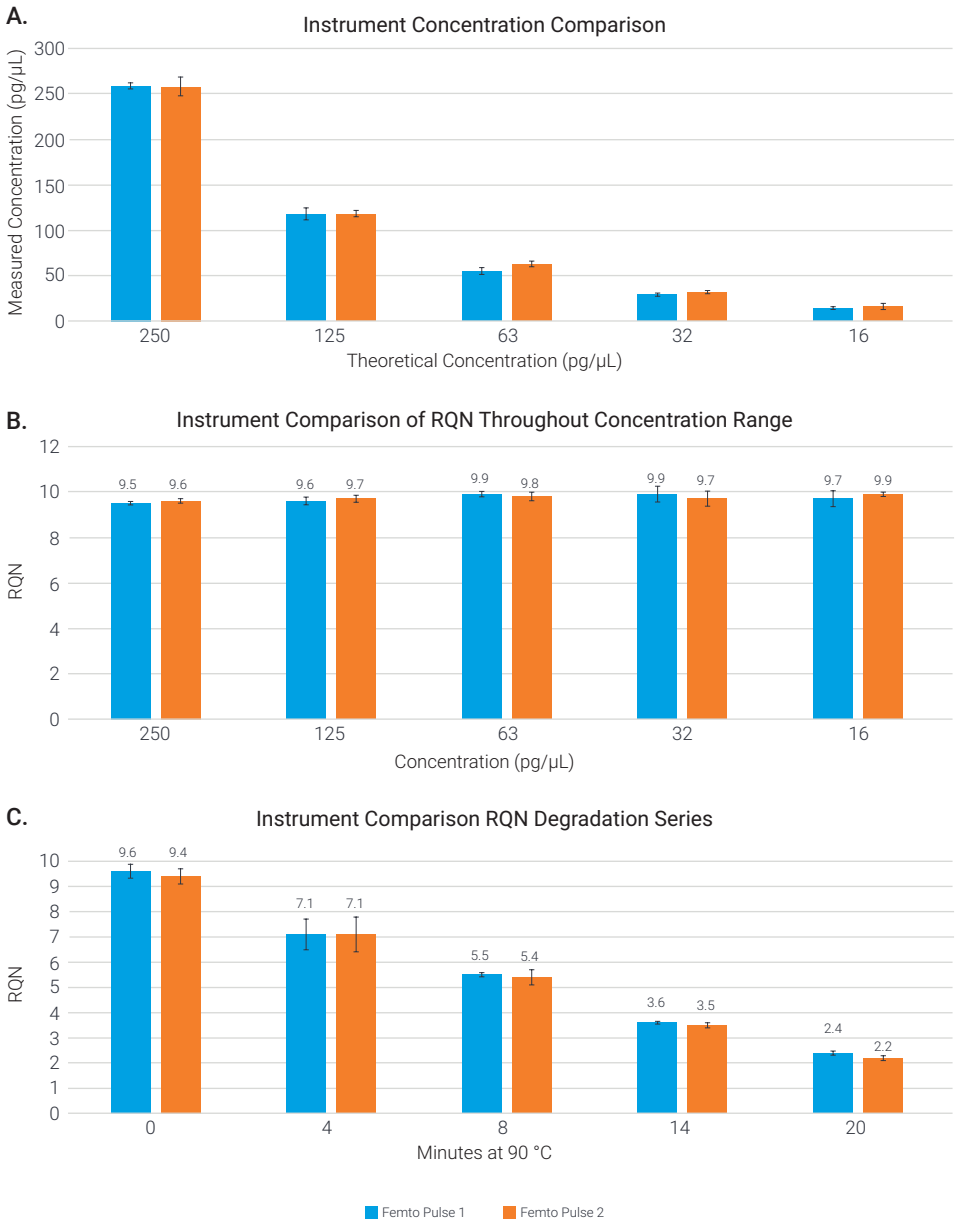


Figure 3. Total RNA concentration and quality metric RQN compared between Agilent Femto Pulse systems with the Ultra Sensitivity RNA kit. (A) Concentration comparison throughout the dilution series with mouse total RNA, n = 6. (B) RQN comparison throughout the dilution series with mouse total RNA, n = 6. (C) RQN comparison through a degradation series with mouse liver RNA, n = 3.

Comparative analysis of RQN with the Fragment Analyzer system

The RQN quality metric was compared throughout a degradation series using the Femto Pulse system with the US RNA kit and the Fragment Analyzer system with both the RNA kit (15 nt) and HS RNA kit (15 nt). Eukaryotic total RNA samples included total RNA tissue from human, mouse and rat samples. Replicates were averaged, resulting in 58 RQN/RQN pairs ranging from 1.1–10. A slope and R^2 value of 1 indicates a perfect linear correlation. A comparison between the Femto Pulse system and the Fragment Analyzer system resulted in a slope of 0.977 with an R^2 value of 0.9875 (Figure 4), confirming the accurate results generated with the Femto Pulse system. Both demonstrated excellent correlation and linear regression with an average difference between data points of ± 0.2 units.

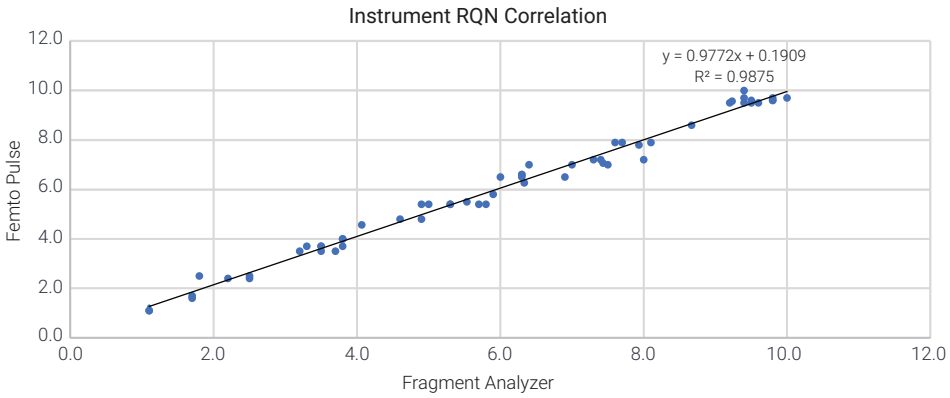


Figure 4. Comparison of RQN from total RNA samples analyzed on the Agilent Femto Pulse system with the Ultra Sensitivity RNA kit and the Agilent Fragment Analyzer system with the RNA kit (15 nt) and the HS RNA kit (15 nt), n = 58.

Conclusion

The Agilent Femto Pulse system and the Ultra Sensitivity RNA kit allow for easy and reliable analysis of total RNA quality and concentration. The Agilent ProSize data analysis software provides an electropherogram and digital gel image for visual inspection of RNA and automatically reports the concentration, ribosomal ratio and quality metric RQN score. The Femto Pulse provided consistent separation of the total RNA throughout the dilution series with sensitivity into the femtogram range. In addition, similar concentrations were reported between instruments, providing reliability from one instrument to another. RNA degradation was easily visualized with the electropherogram and digital gel image, with the RQN reflecting the degree of total RNA degradation. The RQN remained consistent between Femto Pulse instruments and across the dilution and degradation series. The Femto Pulse and Fragment Analyzer capillary electrophoresis instruments reported a high RQN correlation throughout a wide degree of total RNA degradation, allowing for interchangeability and comparison of data between laboratories.

References

1. Genomic DNA sizing and Quality Control on the Agilent Femto Pulse System. *Agilent Technologies application note*, publication number 5994-0516EN, **2019**.
2. cfDNA Separated on the Agilent Femto Pulse System. *Agilent Technologies application note*, publication number 5994-0514EN, **2019**.
3. Genomic DNA Handling and the Agilent Femto Pulse System. *Agilent Technologies application note*, publication number 5994-2723EN, **2020**.
4. Performance of the Agilent 55 and 165 kb BAC Kits on the Agilent Femto Pulse System. *Agilent Technologies application note*, publication number 5994-1102EN, **2020**.
5. Comparison of BAC Fragment Sizing with the Agilent Femto Pulse System and Pulsed-Field Gel Electrophoresis. *Agilent Technologies application note*, publication number 5994-1161EN, **2020**.
6. Comparison of Agilent Femto Pulse System Sizing with Long-Read Sequencing Read Length. *Agilent Technologies application note*, publication number 5994-3078EN, **2021**.
7. Comparison of RIN and RQN for the Agilent Bioanalyzer and Fragment Analyzer Systems. *Agilent Technologies technical overview*, publication number 5994-1869EN, **2020**.

www.agilent.com/genomics/femto-pulse

For Research Use Only. Not for use in diagnostic procedures.
PR7000-8083

This information is subject to change without notice.

Printed in the USA, July 15, 2021
5994-3669EN

Agilent
Trusted Answers