Technical Overview

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Best Practices for Analysis of In Vitro Transcribed (IVT) mRNA

Using the Agilent Fragment Analyzer systems

Introduction

The recent success of in vitro transcription (IVT) mRNA as an emergent platform has brought to the forefront the need for reliable and robust quality control (QC) analysis throughout the workflow. Within the IVT workflow for mRNA vaccine production there are many opportunities for QC that help in the development of a consistent product. The Agilent Fragment Analyzer systems can be utilized for several QC steps at different checkpoints in the mRNA vaccine development workflow.[1] QC steps that can be performed using the Fragment Analyzer include determining the quality and size of the linearized plasmid, size and purity of the IVT mRNA[2,3], length of the poly(A) tail[4], and purity of the final mRNA vaccine product[1].

IVT mRNA yield and quality can be affected by a multitude of factors, including temperature, incubation time, sequence, secondary structure, and mRNA size. This technical overview discusses best handling practices for IVT mRNA and analysis with the Fragment Analyzer systems, including sample handling and quantification tips. Also, we provide guidance for determining the appropriate methods of analysis of IVT mRNA samples and options for optimizing the QC process for different mRNA transcripts.

Experimental

Aliquots of Lambda DNA (Thermo Fisher Scientific, part number SD0021) were PCR amplified using Phusion DNA polymerase to generate templates of sizes ranging from approximately 200 to 6,000 base pairs (bp), shown in Table 1. Following amplification, each PCR reaction was purified using the NucleoSpin Gel and PCR Clean-up kit (Takara Bio, part number 740609.50) and quantified using the Qubit fluorometer (Thermo Fisher Scientific).

The purified PCR products were then used as DNA templates for in vitro transcription. One µg of each template was used to prepare an IVT mRNA sample using the T7 RiboMAX Express Large Scale mRNA Production System (Promega, part number P1320). The IVT mRNA fragments were purified using the mRNA Clean & Concentrator-5 kit (Zymo Research, part number R1013). The final samples were quantified using the NanoDrop One spectrophotometer (Thermo Fisher Scientific), and the size and purity were assessed using the Agilent 5200 and 5300 Fragment Analyzer systems with the Agilent RNA kit (15 nt) (part number DNF-471).[5]

Table 1. List of IVT mRNA samples used in this study and their sequence size.

Reaction Number	Sequence Size (nt)
1	212
2	410
3	493
4	894
5	996
6	1,902
7	2,055
8	3,900
9	4,053
10	5,979

Results and discussion

Mixing

RNA is easily degraded by improper sample handling, such as overmixing and unsuitable storage conditions. To provide guidance on the most efficient method for mixing IVT mRNA with the Agilent RNA Diluent Marker for analysis with the Fragment Analyzer systems, various mixing methods were explored. First, a 2,055 nt sample was prepared in a master mix, mixed by pipetting, then aliquoted onto the sample plate (wells 1 to 5). The same sample was loaded directly onto the plate with the diluent marker and analyzed immediately after heat denaturation with no mixing (wells 6 to 10).

As shown in the digital gel images in Figure 1, the master mixed samples display uniform bands, while the samples that were not mixed show a large variation in peak intensity. This variation will affect the peak height, reported concentration, and percent total calculations from the Fragment Analyzer analysis. This data provides evidence that proper mixing of IVT mRNA samples is necessary for reliable sample analysis.

The Agilent RNA kit (15 nt) for the Fragment Analyzer lists various methods that are acceptable for mixing total RNA samples with the RNA Diluent Marker. To ensure that each of these methods were acceptable for use with IVT mRNA, 996 and 2,055 nt IVT mRNA samples were prepared with the different mixing methods.

In method A, a master mix was prepared by vortexing enough sample and RNA Diluent Marker for aliquoting into five wells. In method B, 22 µL of diluent marker and 2 µL of sample were added to five individual wells of a PCR plate and mixed using a plate vortex (VWR, part number 102093-352) set at 2,000 rpm for two minutes. In method C, the samples and diluent marker were also added to five individual wells but were mixed five times using a pipette set to 20 µL.

The prepared sample plates were heat denatured at 70 °C for 2 minutes, snap cooled at 4 °C, and analyzed on the Fragment Analyzer with the RNA analysis method. The size, concentration, percent total, and peak heights for each sample (2,055 nt shown in Figure 2; 996 nt not shown) did not significantly change between the mixing methods. Importantly, the mixing method did not elicit any degradation or size shifting of the sample. Together, these data indicate that mixing of IVT mRNA samples with the RNA Diluent Marker is crucial for reliable analysis of IVT mRNA, and that any mixing method will provide similar results.

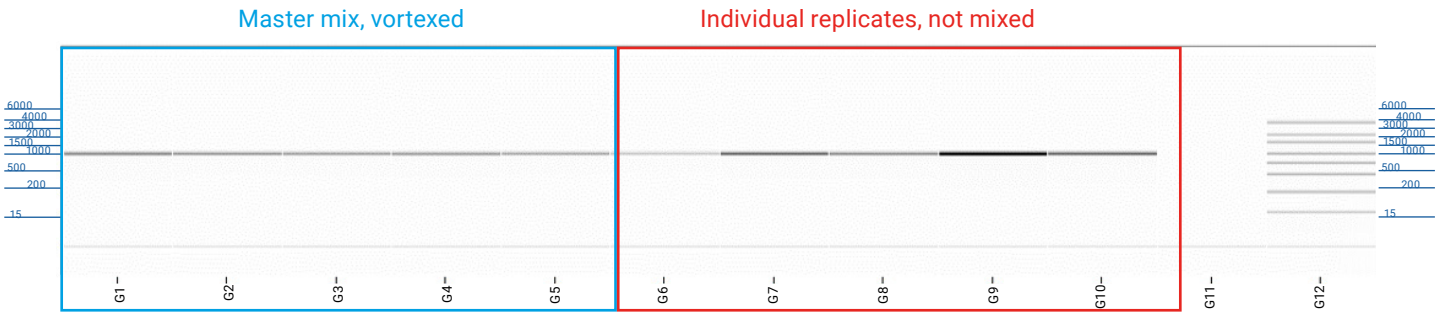


Figure 1. Digital gel image of an IVT mRNA sample on the Agilent 5200 Fragment Analyzer system prepared as a master mix and vortexed (wells 1 to 5, blue outline) compared to samples prepared individually and not mixed (wells 6 to 10, red outline). Samples that were not mixed show clear differences in peak intensity, compared to the uniform bands of the mixed samples (n = 5 samples per method).

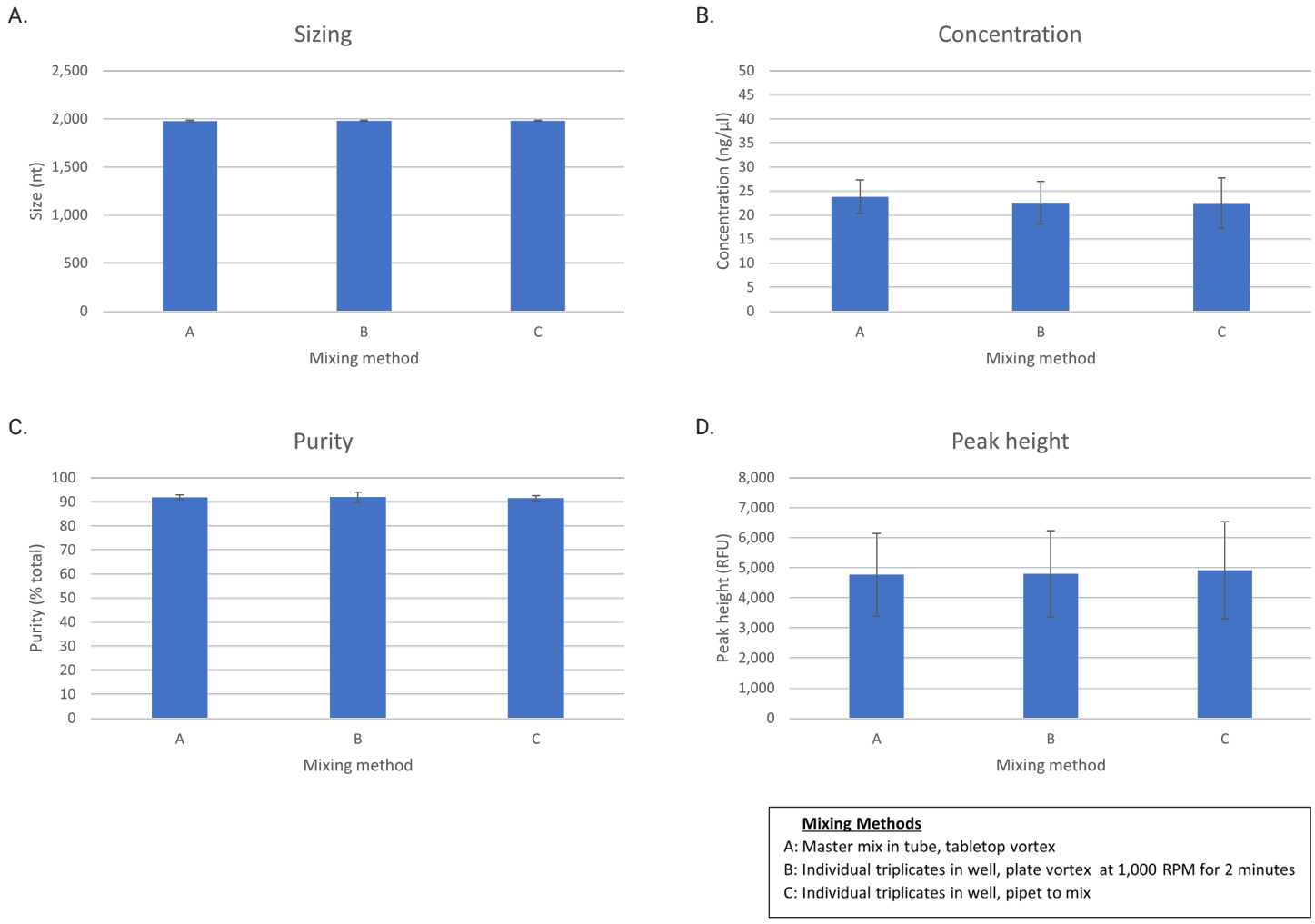
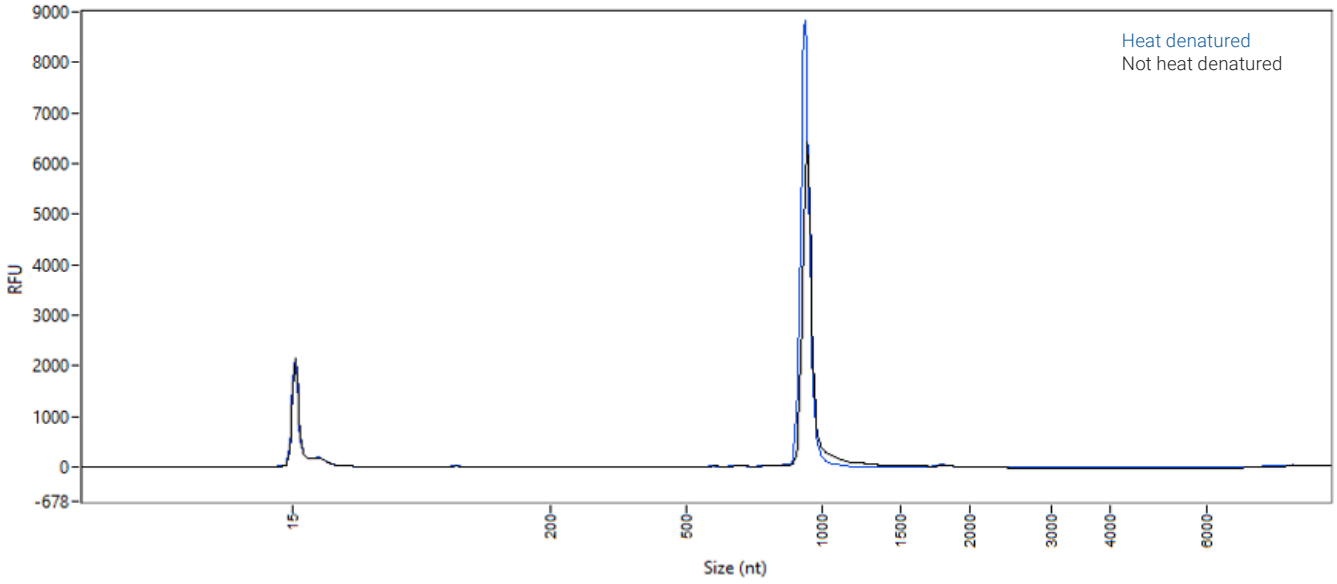


Figure 2. Comparison of different methods of mixing a 2,055 nt IVT mRNA sample with the Agilent RNA Diluent Marker for analysis with the RNA kit on a 5200 Fragment Analyzer system. The A) sample size, B) concentration, C) purity total, and D) peak height was not significantly changed with the different mixing methods (n = 5).

A.



B.

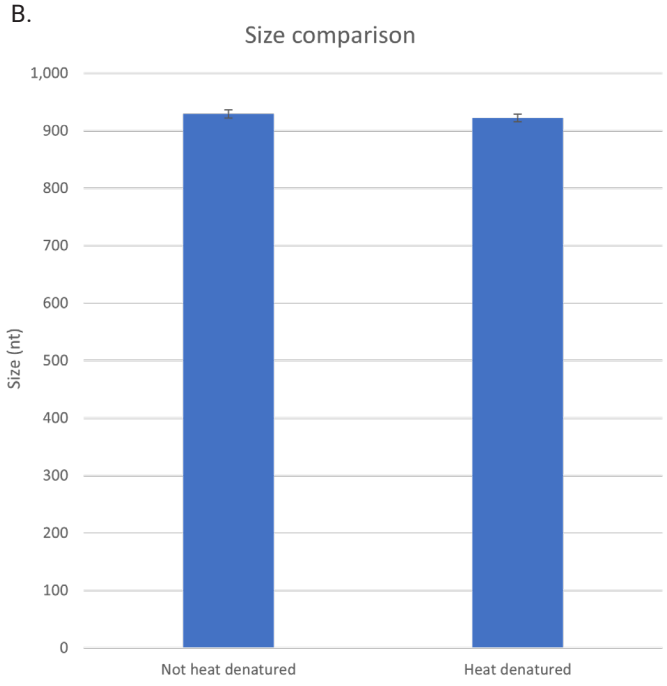


Figure 3. Effect of heat denaturation of IVT mRNA samples analyzed on the Agilent 5300 Fragment Analyzer system. A) Representative electropherogram overlay and B) average fragment size (n = 46) of a 996 nt IVT mRNA sample with and without heat denaturation. Error bars represent standard deviation.

Heat denaturing

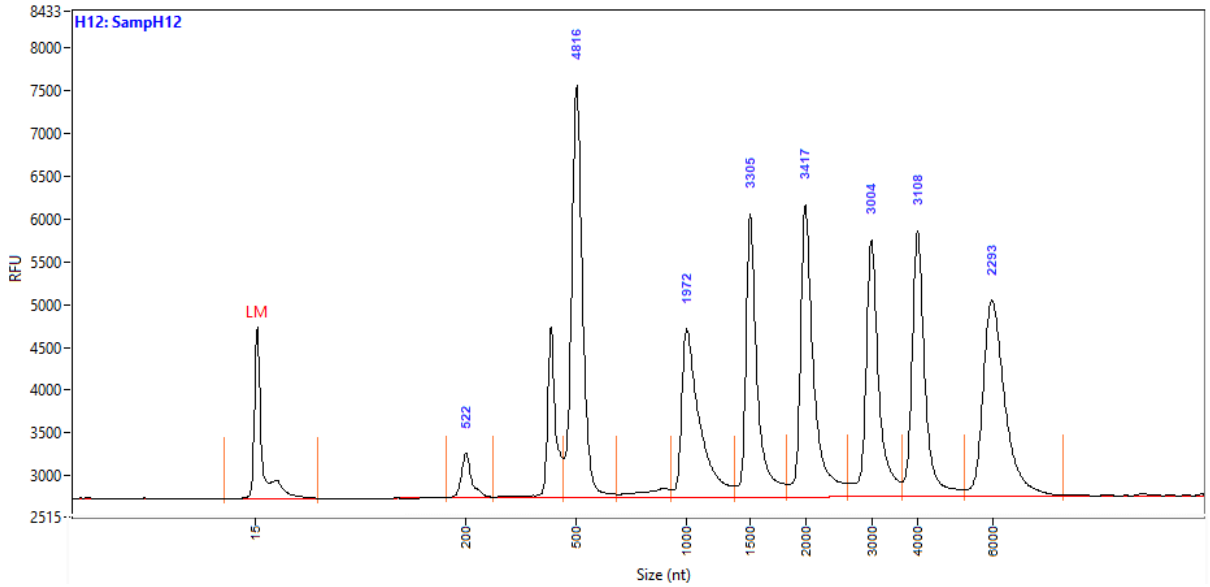
Denaturation is a common method in RNA protocols to eliminate secondary structures present in the RNA. This can be done by applying heat to the samples or mixing them with a chemical denaturant. Analysis of samples with the Fragment Analyzer requires samples to be mixed with the RNA Diluent Marker, which contains 50% formamide, a common storage solvent that protects RNA from degradation by RNases and provides some level of denaturation. Thus, we examined if heat denaturation is also necessary for IVT mRNA analysis with the Fragment Analyzer.

IVT mRNA samples of various sizes and the Agilent RNA Ladder were mixed with the RNA Diluent Marker and analyzed on the Fragment Analyzer before and after heat denaturation at 70 °C for 2 minutes, then snap cooled to 4 °C.

A comparison of the samples before and after heat denaturation indicates that heat denaturation results in a sharper peak. This change in sample distribution may impact peak heights and thus could influence the reported purity of the sample. For example, Figure 3A is an overlay of a 996 nt IVT mRNA sample before and after heat denaturation. In this example, heat denaturation of the sample increased the sharpness of the peak but did not significantly affect the reported percent purity. However, the size of the IVT mRNA sample remains consistent with and without heat denaturation (Figure 3B).

Heat denaturation is required for the RNA Ladder. As shown in Figure 4, heat denaturation of the ladder results in sharper peaks of uniform heights and eliminates secondary structures, such as the split peak seen at 500 nt when the ladder is not heat denatured. Depending on the sample, heat denaturation may or may not be required before analysis with the Fragment Analyzer system. If a secondary structure is seen, the sample can be heat denatured and reanalyzed for best results.

A. Not heat denatured



B. Heat denatured

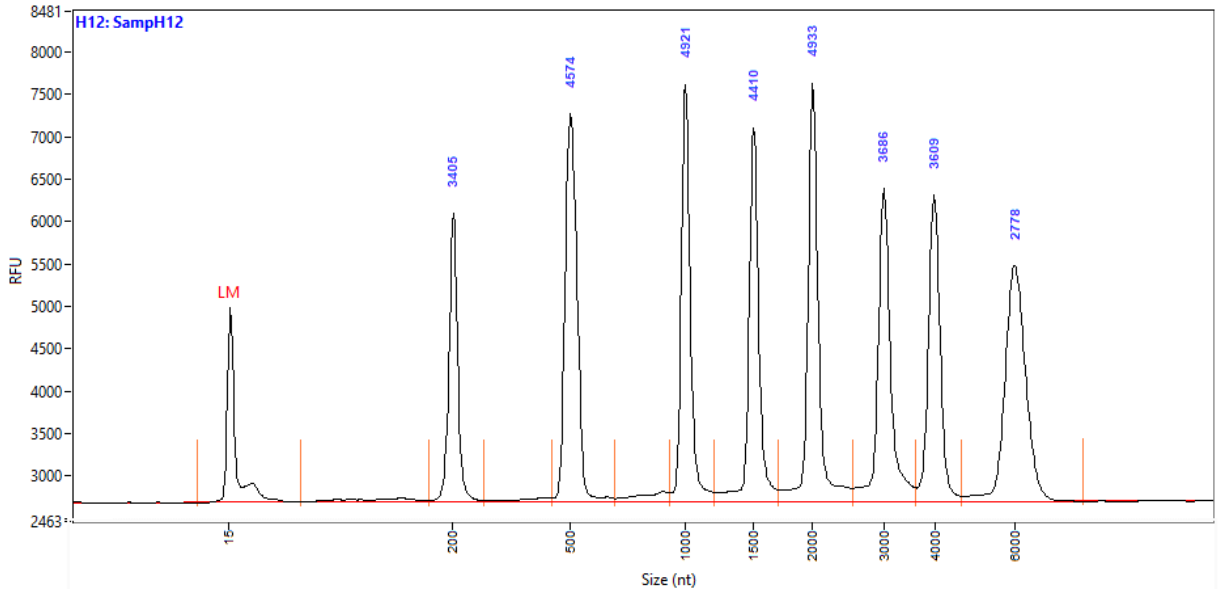


Figure 4. Heat denaturation of the Agilent RNA Ladder is required for accurate sizing analysis with the Agilent 5200 Fragment Analyzer system, as indicated in the electropherogram image of the ladder A) without and B) with heat denaturation. Heat denaturation eliminates secondary structure and results in sharper, more consistent peak heights throughout the size range of the ladder.

Stability

The Fragment Analyzer system can hold up to three 96-well sample plates and can be programmed to run multiple sample rows consecutively. To ensure that IVT mRNA remains stable at room temperature over time, replicates of a 2,055 nt sample were prepared (n = 5) and analyzed with the RNA kit (15 nt) over 10 subsequent runs, with approximately one hour elapsing between the start of each run. No significant change in sample appearance, size, or percent purity was observed over this time (Figure 5). The IVT mRNA samples analyzed were stable at room temperature for 10 hours, providing sufficient time to analyze many rows of samples on the Fragment Analyzer without having to worry about degradation.

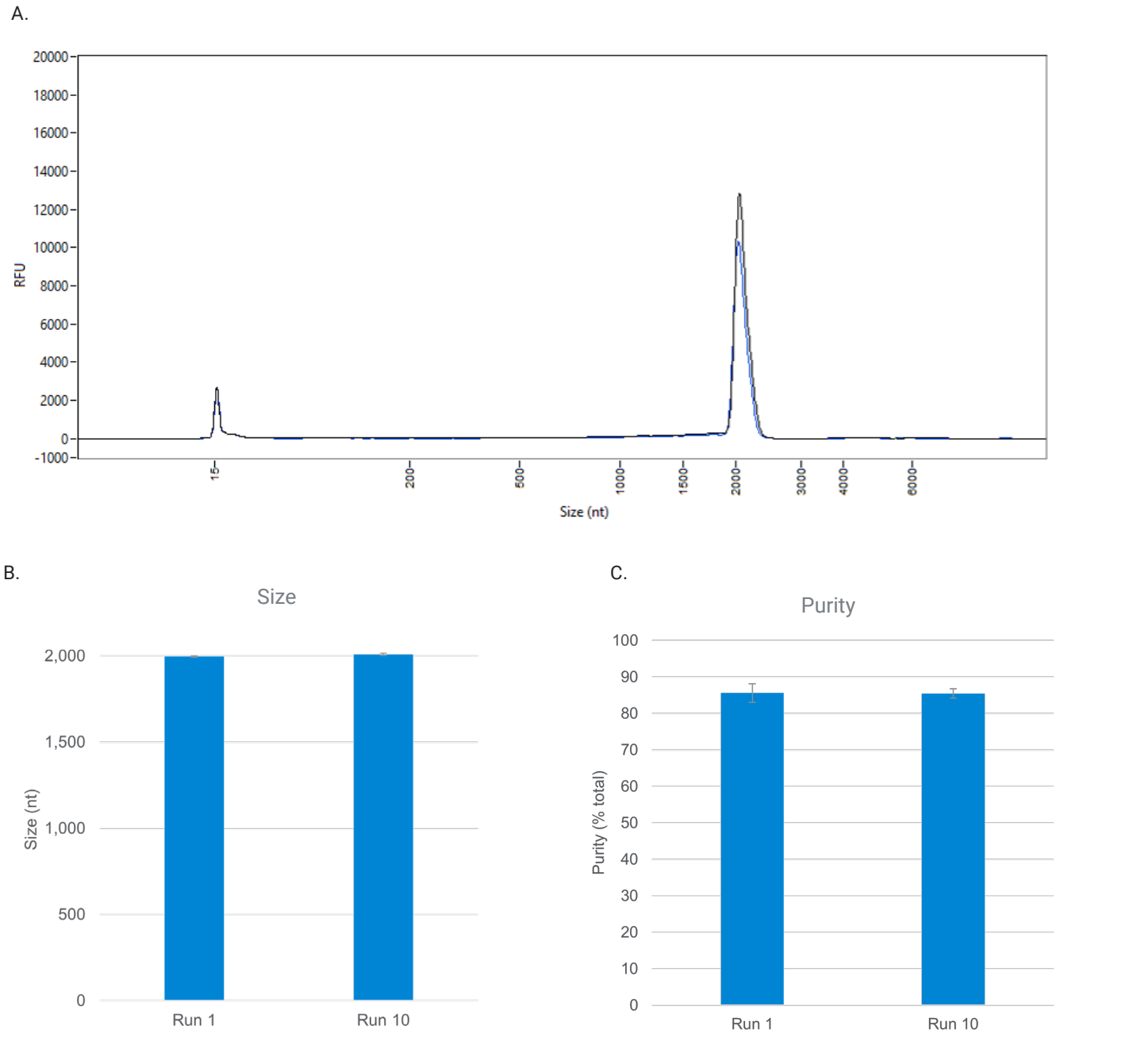


Figure 5. IVT mRNA samples prepared for analysis on an Agilent 5200 Fragment Analyzer system remained stable at room temperature for approximately 10 hours. A representative 2,055 nt IVT mRNA sample was analyzed over 10 subsequent runs, with no significant change in A) sample distribution, B) size, or C) purity, which was assessed using the percent total with a smear range from 1,800 to 2,400 nt.

Instrument reproducibility

To investigate the reproducibility of IVT mRNA analysis, triplicate replicates of IVT mRNA samples of sizes 212, 894, and 1,902 nt were each analyzed across four Agilent Fragment Analyzer systems simultaneously. The average reported size of each fragment is shown in Figure 6, and is consistent between all instruments with a %CV of less than 0.5% for each sample. Also, the size percent error was excellent, with less than 8% error for the 212 nt sample, less than 3% error for the 894 nt sample, and less than 1.5% error for the 1,902 nt sample.

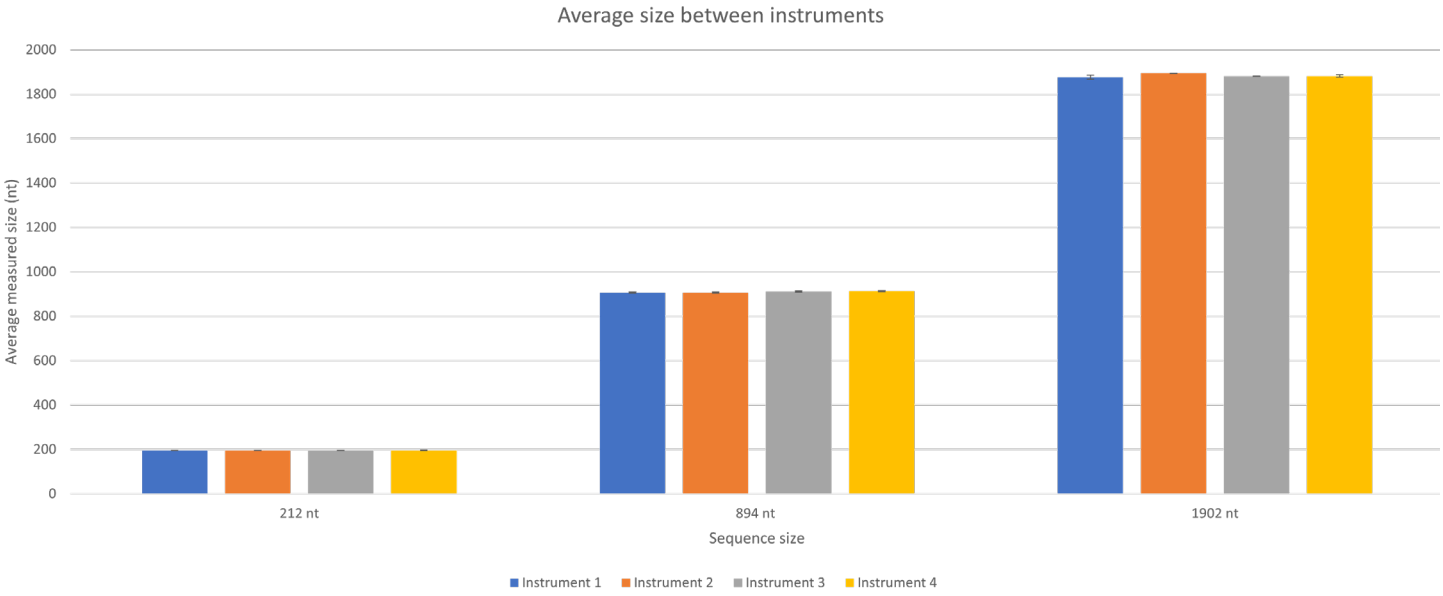


Figure 6. Three IVT mRNA samples of varying sizes were evaluated in triplicate across four Agilent 5200 Fragment Analyzer systems to demonstrate the ability of the instruments to provide reproducible analysis data. The average size of each fragment remained consistent across all instruments tested. Error bars show standard deviation.

IVT mRNA quantification

The RNA kit for the Fragment Analyzer recommends that samples are loaded within a specific concentration range that is necessary for appropriate analysis of the sample. To ensure that samples fit this range, it is important to accurately quantify the sample. Common techniques for quantification of total RNA include either UV-Vis spectrometry or fluorescent methods. However, fluorescent systems such as the Agilent Fragment Analyzer and the Qubit fluorometer (Thermo Fisher Scientific) utilize an intercalating dye that may not bind to IVT mRNA with appropriate affinity to allow for accurate quantification of these samples. To provide guidance for the most accurate quantification of IVT mRNA, UV-Vis and fluorescent methods were compared. Several IVT mRNA samples of varying sizes were quantified with UV-Vis spectrometry using a NanoDrop One spectrophotometer (Thermo Fisher Scientific) and diluted with nuclease-free water to 60, 30, and 15 ng/µL to fit the range of the Fragment Analyzer. The concentration of each sample dilution was confirmed with the NanoDrop. These samples were then evaluated with the Fragment Analyzer and the Qubit for comparison. While the concentration of the samples decreased with the serial dilution as expected, the reported concentration from both the Fragment Analyzer and the Qubit did not correlate with the expected concentration from the Nanodrop. For example, shown in Figure 7 is the reported concentration of a 996 nt sample using the NanoDrop, Qubit, and Fragment Analyzer. Results were consistent among many samples that ranged in size from approximately 100 to 4,000 nt. For each fragment, the measured concentrations from the Qubit and Fragment Analyzer were reproducible and consistent with each other, but were lower than the expected concentration reported by the Nanodrop. Thus, for the most accurate quantification of IVT mRNA, and to determine the concentration at which to load samples onto the Fragment Analyzer, it is recommended to use UV-Vis methods. Also, for the most reliable size and purity analysis with the Fragment Analyzer, it is recommended to optimize the input concentration used. If comparing multiple replicates, the best practice would be to ensure that all samples are at the same concentration before loading them onto the instrument.

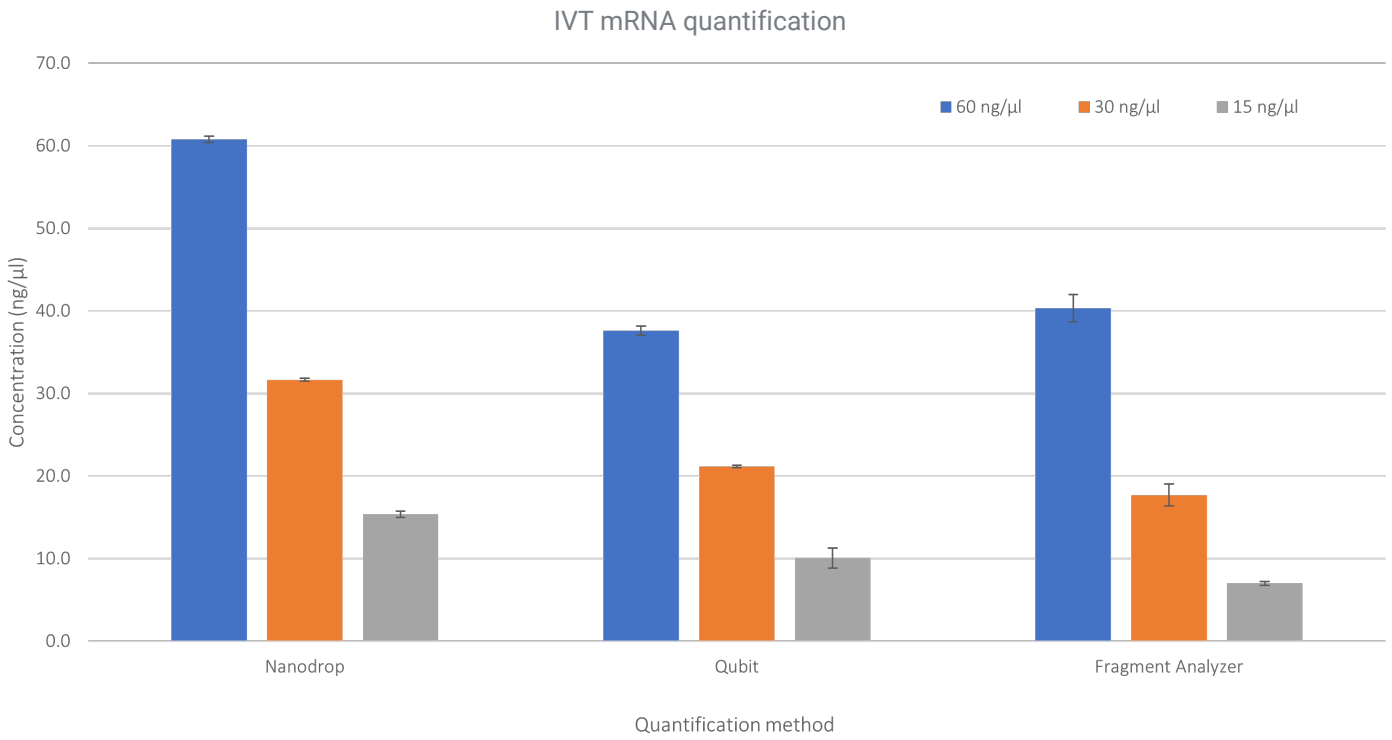


Figure 7. Reported concentration of a 996 nt IVT mRNA sample using the NanoDrop, Qubit, and Agilent 5200 Fragment Analyzer system.

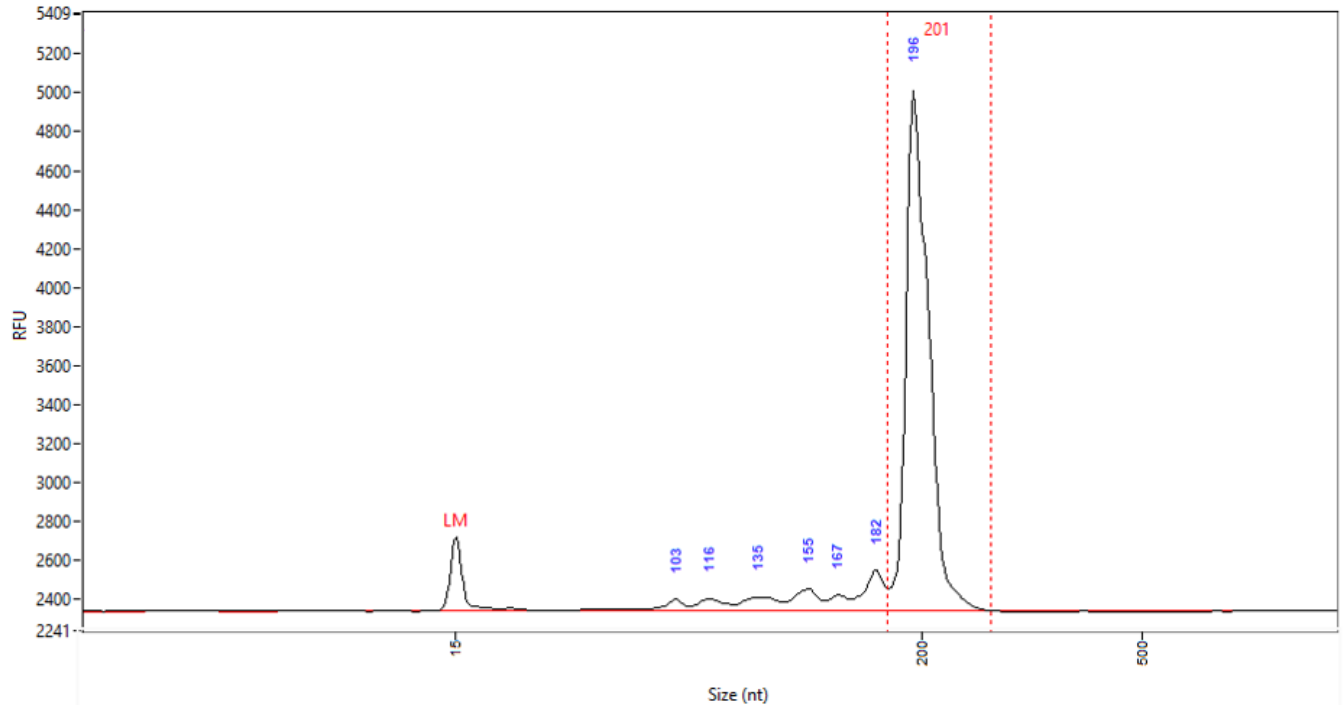
Concentration range

The RNA kit (15 nt) for the Fragment Analyzer system was designed for analysis of total RNA smears covering a large concentration range, which has been further refined for IVT mRNA fragments. Since IVT mRNA fragments are seen as sharp peaks, the same concentration range does not apply. The same sample concentration will give larger peak heights, and, if too high, may cause cross-talk in the surrounding wells. The fragment peak height should be optimized for accurate analysis. The maximum peak height recommendation is approximately 24,000 RFU to avoid cross-talk. If samples show a higher peak height, they should be diluted with nuclease-free water and the analysis run repeated.

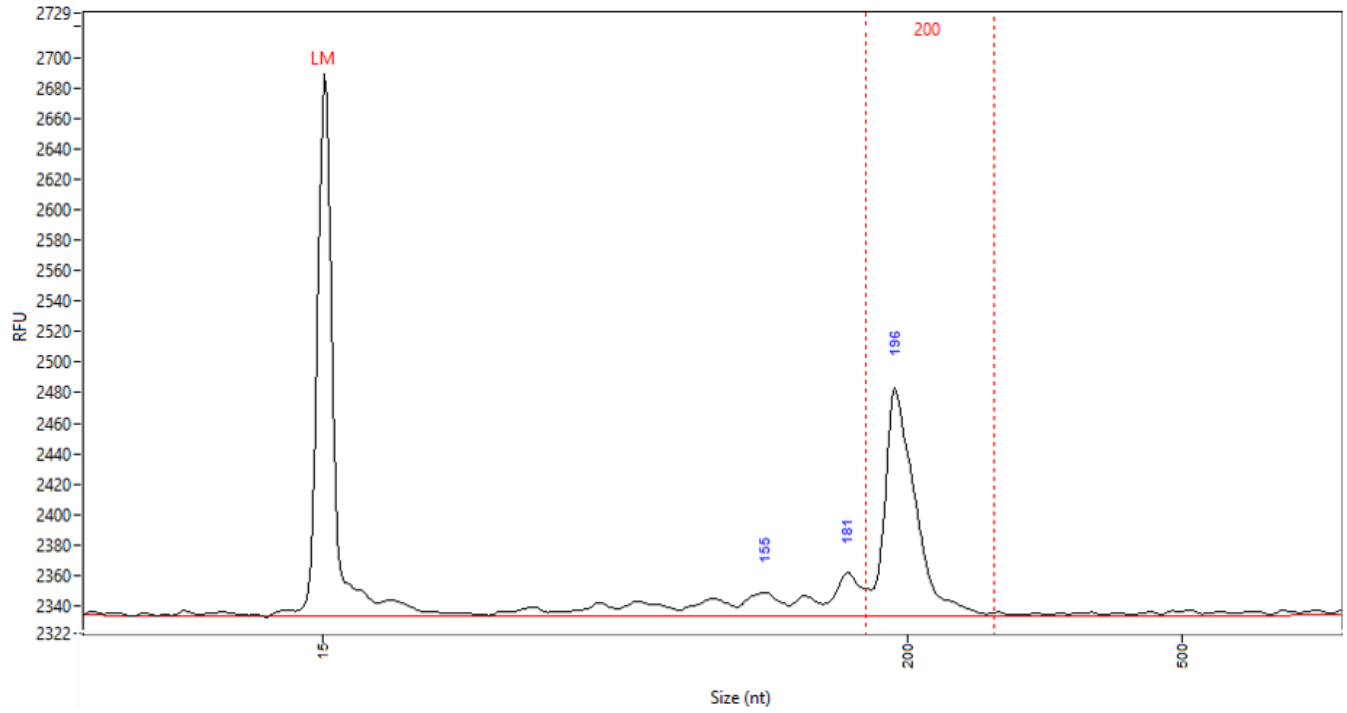
In addition, one of the primary goals of IVT mRNA analysis with the Fragment Analyzer is to examine the sample for percent purity. Thus, the sample must be run at a high enough concentration to allow for the reliable detection of any small impurities present in the sample. If the concentration tested is too low, these impurity peaks may be too small to be successfully integrated in the electropherogram, and the reported percent purity will be overexaggerated. Within the Agilent ProSize data analysis software, the minimum peak height can be adjusted to ensure that all peaks are successfully integrated, thus allowing for better analysis of sample purity.

As an example, shown in Figure 8 is a 212 nt IVT mRNA sample that was analyzed at various concentrations. At the highest concentration, 100 ng/µL, the main fragment is displayed as a large peak at approximately 200 nt, with many smaller impurity peaks to the left (Figure 8A). As the sample concentration decreases, the number of impurity peaks that are automatically integrated into the analysis decreases, as indicated by the peaks with a number above them. The smaller peaks without a number are not being integrated into the analysis as the signal-to-noise-ratio is too low, and the peaks could be considered noise (Figure 8B). At the lower end of the concentration range, 3 ng/µL, the main peak height is decreased, and the small impurity peaks are not visualized (Figure 8C). It should be noted that IVT mRNA fragments can run differently based on the sequence and composition of the sample, and what works well for one transcript will not necessarily work for all. Thus, it is best practice to optimize the input concentration for each type of sample to be analyzed. As a guideline, it is recommended to start sample analysis within the recommended range of 1 to 100 ng/µL[2,5] and optimize the sample concentration to fit the guidelines stated here.

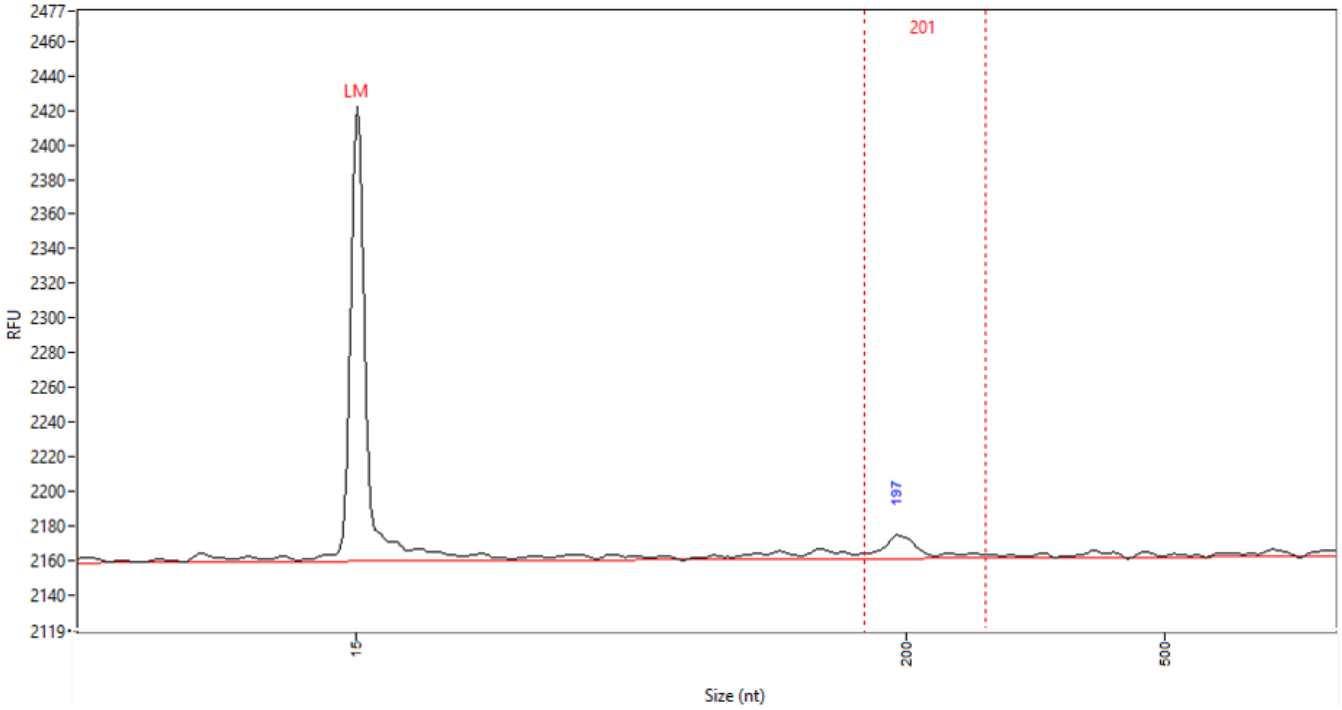
A.


RFU
Size (nt)
LM
103
116
135
155
167
182
196
201
15
200
500

B.


RFU
Size (nt)
LM
155
181
196
200
15
200
500

C.



D.

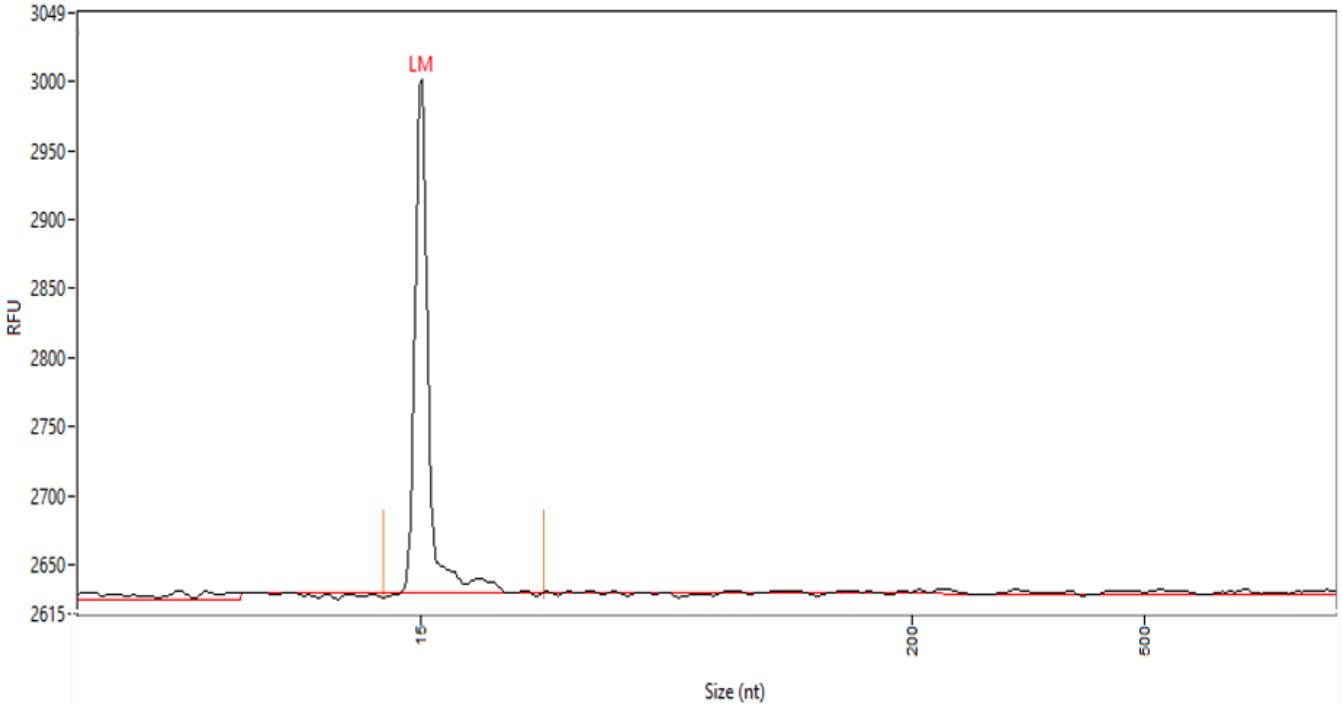


Figure 8. Serial dilution of a 212 nt IVT mRNA fragment analyzed on an Agilent 5200 Fragment Analyzer system. Shown are electropherogram images of the sample at concentrations of A) 100, B) 12.5, and C) 1.56 ng/µL. D) A sample well with with diluent marker only, used as a negative control.

Conclusion

The Agilent RNA kit (15 nt) for the Agilent Fragment Analyzer systems can be utilized in many steps of the IVT mRNA vaccine development workflow for size and purity assessment. IVT mRNA fragments may behave differently in automated electrophoresis due to factors such as size, sequence, and composition. Thus, this technical overview highlights sample handling techniques and guidelines that should be utilized for accurate and reliable IVT mRNA analysis with the Fragment Analyzer.

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www.agilent.com/genomics/fragment-analyzer

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