Technical Overview

Genomics

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High-Sensitivity IVT mRNA Analysis Using the Agilent Fragment Analyzer Systems

Introduction

Recently, quality analysis of in vitro transcribed (IVT) mRNA has become an essential part of biotherapeutic workflows, such as vaccine development. The Agilent Fragment Analyzer systems offer a variety of RNA analysis kits to aid in the assessment of some of the key critical quality attributes (CQAs) assessed throughout these workflows, including size, integrity, and purity of the IVT mRNA. Among the RNA kits for the Fragment Analyzer is the Agilent High-Sensitivity (HS) RNA kit (15 nt) (part number DNF-472). New methods for assessment of IVT mRNA on the Fragment Analyzer systems have been developed for the HS RNA kit, expanding the options beyond the original methods for total RNA and ribo-depleted RNA.[1] Total RNA and IVT mRNA each have unique analysis requirements and different profiles necessitating distinct methods for each. Electrophoresis of IVT mRNA depicts the sample as a single sharp peak, with any impurities or degradation flanking the peak. In its use as a biotherapeutic, CQAs including purity and sizing of the main peak are evaluated. Due to differences in RNA sample profiles, analysis requirements, and concentration ranges, new HS RNA methods were developed to specifically focus on the growing demands for IVT mRNA therapeutics.

The new methods available for the HS RNA kit provide optimized injection and separation conditions for IVT mRNA samples. The optimized conditions can accommodate samples up to 9,000 nt over a broad concentration range, while still using the same HS RNA kit and reagents (methods A and B).[2] The analytical specifications for the expanded HS RNA kit for IVT mRNA analysis methods, in addition to total RNA and ribo-depleted RNA, are summarized in Table 1. The HS IVT mRNA low-concentration method (method A) is for samples of low concentration, with an input range of 500 to 2,500 pg/µL. The HS IVT mRNA mid-concentration method (method B) is for mid-range concentration samples from 2,500 to 10,000 pg/µL, bridging the gap between the standard sensitivity Agilent RNA kit (15 nt) (part number DNF-471) and the HS RNA kit method A range. Both methods are used for analysis of smaller IVT mRNAs from 200 to 6,000 nt. Additional extended methods are available for the sizing of long IVT mRNAs from 500 to 9,000 nt for both the low- and mid-concentration ranges (methods AE and BE, respectively). These methods use a longer separation time and an alternative ladder for analysis. In addition to the extended sizing range, these methods offer high resolution of closely sized fragments throughout the sizing range of the kit. This technical overview details the different methods available for the analysis of IVT RNA using the HS RNA kit on the Fragment Analyzer.

Experimental

Reference IVT RNA samples (GenScript Biotech special order for 1,808, 4,305, and 9,000 nt) were prepared in nuclease-free water at 10 ng/µL, and concentration was confirmed with a NanoDrop spectrophotometer. Further dilutions were prepared to fit the concentration ranges of the different methods (Table 1). Multiple replicates of all samples were assessed using the HS RNA kit on the Agilent 5200 and 5300 Fragment Analyzer systems equipped with 12, 48, and 96 short capillary arrays. The method was chosen to fit the sizing and concentration ranges of the kits. The 1,808 and 4,305 nt samples used methods A and B, while all three samples were analyzed with the extended methods AE and BE. Samples of lower concentrations from 15 to 2,500 pg/µL used the longer injection in method A, and concentrations from 2,500 to 10,000 pg/µL used the shorter injection time in method B. For methods A and B, used the Agilent HS RNA Ladder (part number DNF-386-U015) was heat denatured at 70 °C for 2 minutes and diluted to 2 ng/µL with nuclease-free water before running, according to the quick guide.[2] The extended methods AE and BE used an alternative RNA marker from Lonza (part number 50575) in place of the Agilent HS RNA Ladder. The Lonza RNA marker was diluted to 25 ng/µL and concentration was confirmed with the Nanodrop. Then, it was heat denatured at 70 °C for 2 minutes and diluted to 2 ng/µL with nuclease-free water before addition to the plate.

Results and discussion

IVT mRNA analysis with the Fragment Analyzer systems and HS RNA kit

The HS RNA kit for the Fragment Analyzer has a sizing range of 200 to 6,000 nt. Several methods can be used with the kit to enable assessment of IVT mRNA samples across a broad concentration range. Following separation, the samples are automatically analyzed and can be visualized as both a digital gel image and a detailed electropherogram. To demonstrate the capabilities of the HS RNA kit and the IVT mRNA methods A and B, reference samples of 1,808 and 4,305 nt were analyzed at different concentrations, covering the ranges of both methods. Method A is used for low input concentration ranges from 500 to 2,500 pg/µL, while method B is for mid-range concentrations from 2,500 to 10,000 pg/µL. The electropherogram overlays in Figure 1 highlight the similar sizing achieved across the concentration ranges of both methods A and B.

Results of the average size of each IVT mRNA sample across the concentration ranges of both methods A and B display an excellent linear trendline and Y-equation with slopes below 0.01. A slope of 0 indicates no change in sizing over the concentration range. The slope of the 1,808 nt IVT mRNA was 0.0092, while the slope of the 4,305 nt sample was close to 0, at 0.0012, indicating excellent cohesion between the low- and mid-concentration ranges of the two methods (Figure 1E). This data demonstrates that the IVT mRNA methods A and B for the HS RNA kit both give similar sizing data for IVT mRNA samples across a broad concentration range, enabling researchers to achieve reliable data for a variety of samples with a single kit.

Table 1. Analytical specifications of the Agilent HS RNA kit (15 nt) and associated methods used for analysis of total RNA, ribo-depleted RNA, and IVT mRNA with the Agilent Fragment Analyzer systems.

Reagent Kit	HS RNA kit (15 nt) (p/n DNF-472)					
Application	Total RNA	Ribo-depleted RNA	IVT mRNA			
Method	DNA-472T33 - HS Total RNA 15 nt	DNF-472M33 - HS mRNA 15 nt	DNF-472A33 - HS IVT mRNA Low Concentration (method A)	DNF-472AE33 – 4kV HS IVT mRNA Extended Low Concentration (method AE)	DNF-472B33 - HS IVT mRNA Mid Concentration (method B)	DNF-472BE33 – 4kV HS IVT mRNA Extended Mid Concentration (method BE)
Size Range	200–6,000 nt	200–6,000 nt	200–6,000 nt	500–9,000 nt	200–6,000 nt	500–9,000 nt
Concentration Range	50–5,000 pg/µL	500–5,000 pg/µL	500–2,500 pg/µL	500–2,500 pg/µL	2,500–10,000 pg/µL	2,500–10,000 pg/µL
Sensitivity	15 pg/µL	250 pg/µL	15 pg/µL	15 pg/µL	NA	NA
Injection time	150 s	200 s	150 s	150 s	50 s	50 s
Separation Time	40 min	40 min	45 min	90 min	45 min	90 min
Sizing Accuracy (% Error)	20%	20%	15%	15%	15%	15%
Sizing Precision (% CV)	20%	20%	< 10%	< 10%	< 5%	< 5%
Qualitative Range	50–5,000 pg/µL	500–5,000 pg/µL	NA	NA	NA	NA
Quantification Accuracy (% Error)	30%	30%	NA	NA	NA	NA
Quantification Precision (%CV)	20%	20%	NA	NA	NA	NA

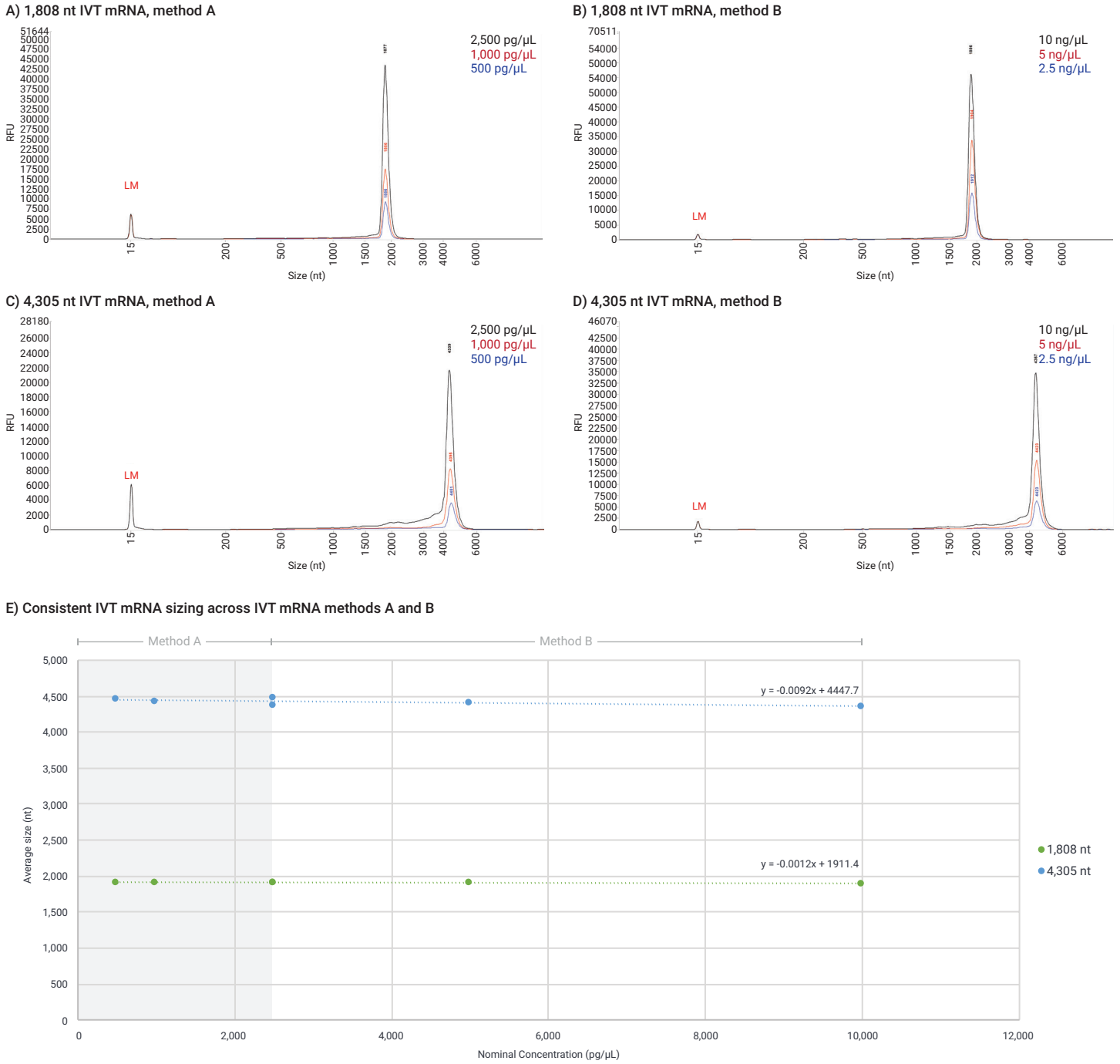


Figure 1. Reference IVT mRNA samples were analyzed on the Agilent Fragment Analyzer systems using the Agilent HS RNA kit with the optimized IVT mRNA methods. Overlay electropherograms of 1,808 nt IVT mRNA with A) method A (n = 14) and B) method B (n = 20 to 25), and the 4,305 nt IVT mRNA with C) method A (n = 22) and D) method B (n = 30 to 36). E) Analysis of the average size of each sample across the concentration ranges of both methods. Each sample concentration was run in multiple replicates on the 5200 and 5300 Fragment Analyzer systems.

Sizing accuracy

To evaluate the sizing accuracy of the methods, different concentrations of the reference samples were analyzed in multiple replicates and compared to their known sizes. Figure 2 summarizes the sizing accuracy of each sample. For the low-concentration method A, the 1,808 and 4,305 nt samples displayed an average accuracy of 5.6% error or less. Comparable results were achieved with the mid-concentration method B, with all samples showing an accuracy of 6.1% error or less. Samples run with both methods A and B were well below the sizing accuracy for IVT mRNA with the HS RNA kit of ± 15% error. Importantly, the longer injection time of method B did not affect the sizing accuracy of the IVT mRNA samples compared to the shorter injection time of method A. Across the concentration range of both samples, the sizing accuracy was well within the specifications of the IVT mRNA methods for the HS RNA kit.

Sizing precision

The HS RNA kit quick guide for IVT mRNA[2] states that the sizing precision for the low-concentration method A is 10% CV, while the mid-range concentration method B is 5% CV. To demonstrate this, two reference samples were assessed at different concentrations covering the ranges of the methods. As shown in Figure 3, the average precision of each sample was less than 3.2% CV for either method, which is well below the kit specifications, with method B displaying slightly lower values than method A. The slight difference may be due to the longer injection time that was used for method A to detect lower concentrations. This is evidenced by the difference in the 2,500 pg/µL sample, which was analyzed with both methods. For example, the sizing precision of the 1,808 nt sample at 2,500 pg/µL was 1.85% CV with method A, and 0.77% CV with method B. Overall, both methods showed excellent precision at all concentrations, with method B displaying slightly lower values.

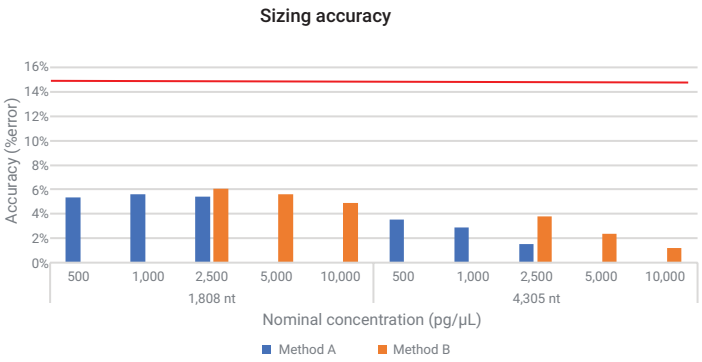


Figure 2. Sizing accuracy of the 1,808 and 4,305 nt IVT mRNA samples at different input concentrations analyzed on the Agilent Fragment Analyzer systems using the Agilent HS RNA kit with methods A and B. All samples were below the kit specification of 15% (red line). Each sample concentration was run in multiple replicates on the 5200 and 5300 Fragment Analyzer systems. (*n* = 14 to 36 replicates per method, size, and concentration.)

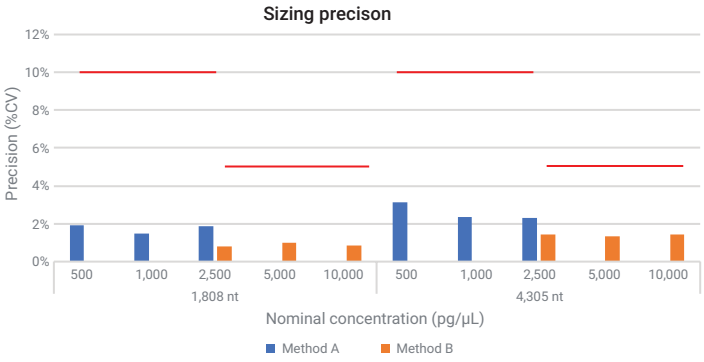


Figure 3. Sizing precision of the 1,808 and 4,305 nt IVT mRNA samples analyzed on the Agilent Fragment Analyzer systems using the Agilent HS RNA kit with IVT mRNA methods A and B. All sample sizes and concentrations were below the kit specification of 10% CV for method A and 5% CV for method B (red lines). Each sample concentration was run in multiple replicates on the 5200 and 5300 Fragment Analyzer systems. (*n* = 14 to 36 replicates per method, size, and concentration.)

Sensitivity and purity assessment

A CQA for IVT mRNA is the percent purity of the IVT mRNA fragment. This can be assessed using the Agilent ProSize data analysis software smear analysis function to identify the percentage of the sample that is composed of the main peak compared to any impurities or degradation. For this analysis, it is necessary to load the sample at a concentration that is high enough for detection of even small amounts of impurities while staying under the recommended RFU value of less than 60,000 to avoid overloading the system.

The HS IVT mRNA low-concentration method A is for samples of low concentration, with an input range of 500 to 2,500 pg/µL to allow for impurity detection. The sensitivity of the method allows for a limit of detection down to 15 pg/µL with consistent sizing, as shown in Figure 4. However, when the total sample concentration is below 500 pg/µL, small impurity peaks may not be detected, impacting percent purity analysis.

As shown in Figure 5, the percent purity is more variable at the lower end of the sensitivity range of the kit, indicated by the %CV values at about 10% or greater for the 1,800 sample at 15 pg/µL and the 4,305 sample at 15 and 30 pg/µL. Within the concentration range of the kit, 500 to 2,500 pg/µL, the %CV of the purity assessment is below 4% for each sample size, highlighting the excellent integrity assessment that can be achieved with the Fragment Analyzer.

For reliable assessment of the impurities within a sample, it is recommended that the method is run with a concentration that gives a peak height of greater than 15,000 RFU, but no more than 60,000 RFU, for the main fragment. However, since all IVT mRNA samples have different sequences, compositions, and modifications that can affect purity, it is important to assess each sample individually and may be necessary to optimize the method or concentration to fit the needs of specific samples.

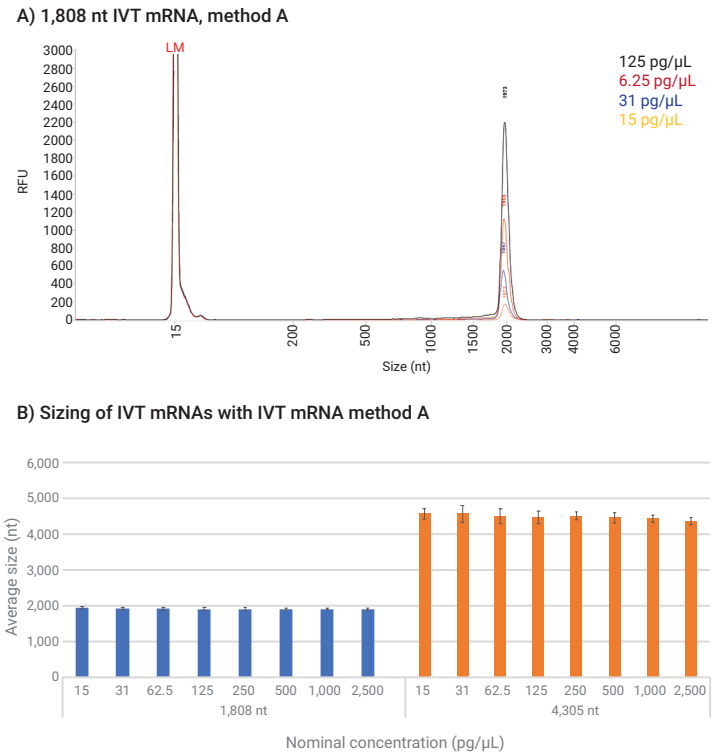


Figure 4. Sensitivity of the Agilent Fragment Analyzer for assessment of IVT mRNA using the Agilent HS RNA kit and the low-concentration method A. A) Electropherogram overlay of 1,800 nt sample, from 15 to 125 pg/µL. B) Average size of the IVT mRNA samples across the concentration range of 15 to 2,500 pg/µL. (n = 14 to 48 replicates per method, size, and concentration.)

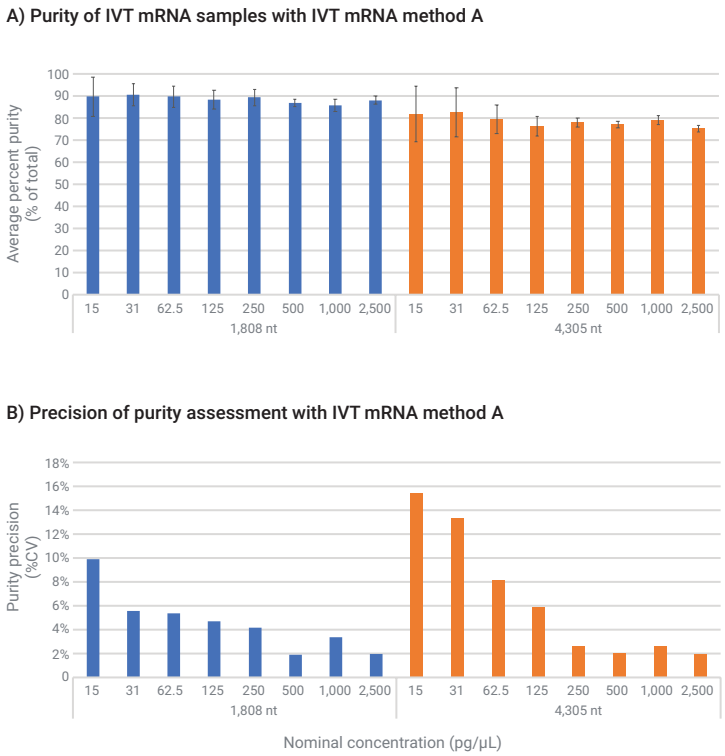


Figure 5. Purity of IVT mRNA samples at different concentrations assessed on the Agilent Fragment Analyzer using the Agilent HS RNA kit and the IVT mRNA low-concentration method A. A) Percent purity. B) Precision of the purity analysis. (n = 14 to 52 replicates per concentration.)

Extended method sizing accuracy and precision

Extended methods AE and BE, for IVT mRNA samples with an expected size larger than 6,000 nt, have been developed, covering the same concentration ranges as methods A and B. These methods use a longer separation time and use an alternative ladder for sizing of samples from 500 through 9,000 nt. In addition, methods AE and BE can provide higher separation resolution of closely sized fragments. To highlight the larger sizing range of these methods, as well as to demonstrate the capabilities of the extended methods for smaller sizes, the 1,808, 4,305, and a 9,000 nt IVT mRNA reference samples were analyzed on the HS RNA kit using the extended methods AE and BE. An overlay of the 9,000 nt IVT mRNA sample is shown in Figure 6A and 6B. Like methods A and B, all samples displayed excellent sizing analysis, with consistent sizing across the entire concentration range for both methods AE and BE, as demonstrated with slopes close to zero (Figure 6C).

Sizing accuracy evaluation of the 1,808, 4,205, and 9,000 nt samples with the extended low-concentration method AE were below 6.2% error. Comparable sizing accuracy results were achieved with the extended mid-concentration method BE, with all samples showing a percent error of less than 7.5% (Figure 7A). The extended separation methods gave similar sizing accuracy to the normal methods. In addition, all samples showed excellent sizing precision, with the samples at each concentration having a %CV of less than 6.6% with the longer injection time of method AE, and less than 2.4% for method BE that has the shorter injection time (Figure 7B).

All samples were well within the kit specification for sizing accuracy and precision using each of the IVT mRNA methods for the HS RNA kit, with method BE displaying slightly better precision. Together, these results highlight the capabilities of the HS RNA kit to analyze IVT mRNAs over a broad range, from 200 to 9,000 nt in length and 500 to 10,000 pg/µL in concentration.

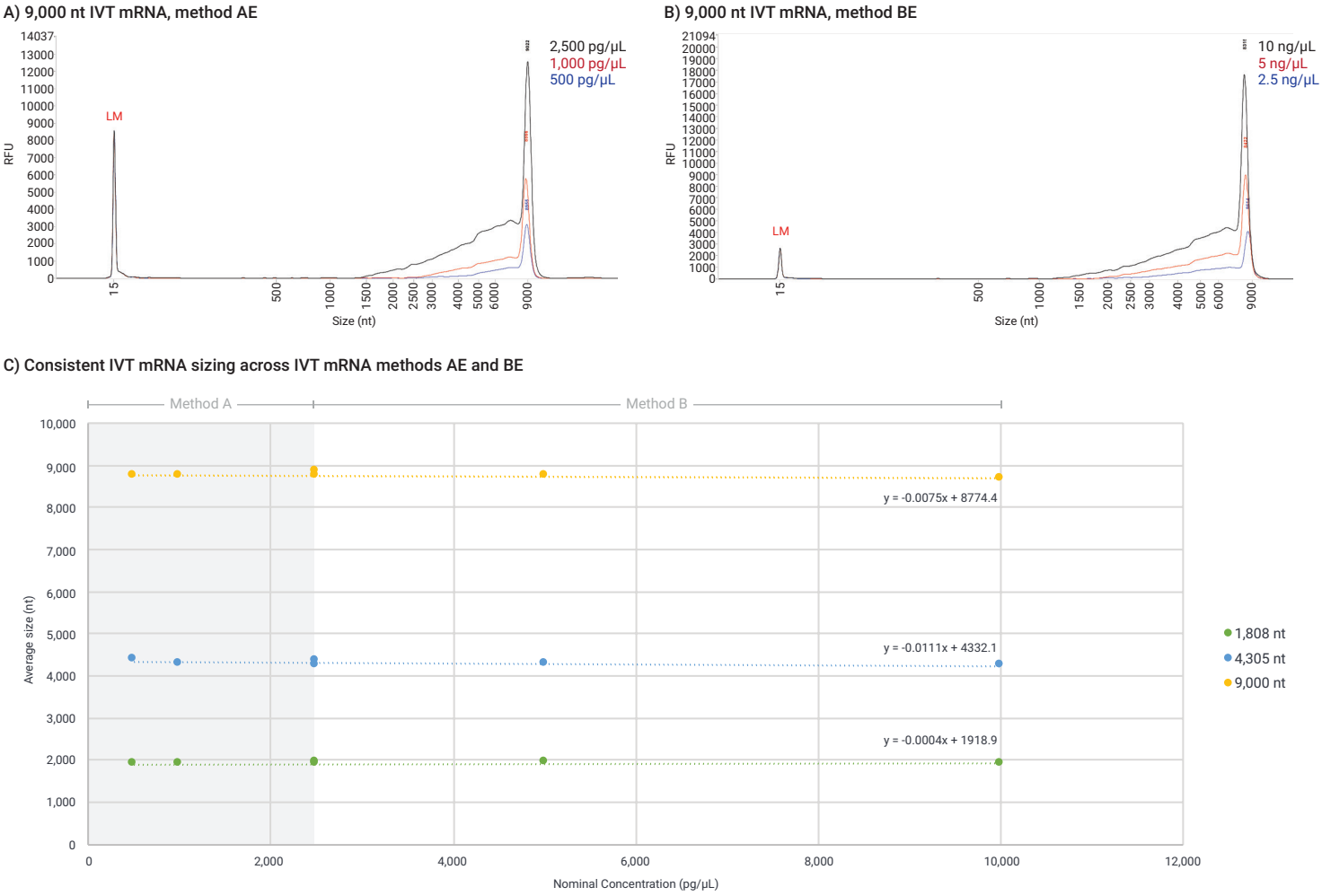
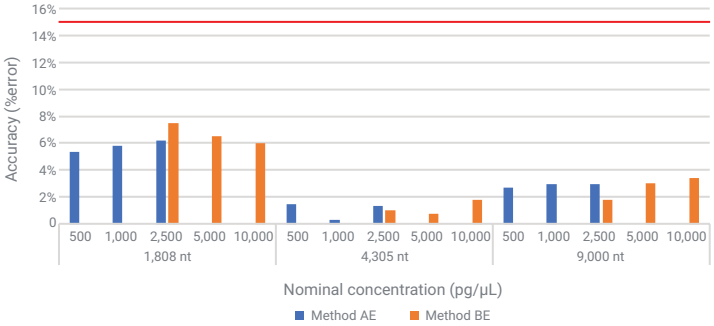


Figure 6. 1,808, 4,305, and 9,000 nt IVT mRNA samples were analyzed on the Agilent Fragment Analyzer systems using the Agilent HS RNA kit with the extended methods AE and BE. Overlay electropherogram of the 9,000 nt sample assessed with A) method AE and B) method BE. C) Sizing analysis across the range of both methods AE and BE. (*n* = 10 to 34 replicates per size and method.)

A) Sizing accuracy of extended IVT mRNA methods AE and BE



B) Sizing precision of extended IVT mRNA methods AE and BE

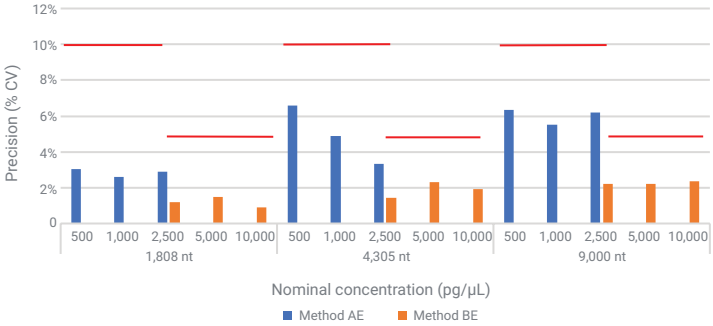


Figure 7. The A) average sizing accuracy and B) sizing precision of three IVT mRNA samples at different concentrations were assessed on the Agilent Fragment Analyzer system using the Agilent HS RNA kit with the extended methods AE and BE. Each sample concentration was run in multiple replicates on the 5200 and 5300 Fragment Analyzer systems. (n = 10 to 34 replicates per size and method.)

Conclusion

The Agilent HS RNA kit for the Agilent Fragment Analyzer systems is used for IVT mRNA analysis of samples from 200 to 9,000 nt. Several methods were developed to separate the large sizing range while also incorporating an expansive concentration range. Methods A and B are for analysis of 200 to 6,000 nt IVT mRNA, covering a low-range concentration of 500 to 2,500 pg/µL with method A, and a mid-range concentration of 2,500 to 10,000 pg/µL with method B. Additionally, the kit offers extended methods for analysis of longer samples up to 9,000 nt (methods AE and BE) while encompassing the same concentration ranges. Together, the new methods encompass broad sizing and concentration ranges suitable for sizing, integrity, and purity analysis of a wide variety of IVT mRNA samples with a single kit using the Agilent Fragment Analyzer systems.

References

1. Agilent DNF-472 (15 nt) HS RNA Kit - Total RNA and ribo-depleted RNA. *Agilent Technologies quick guide*, publication number SD-AT000132, **2024**
2. Agilent DNF-472 (15 nt) HS RNA Kit - IVT mRNA. *Agilent Technologies quick guide*, publication number D0117260, **2024**

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