Technical Overview

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Best Practices for Analysis of IVT mRNA using the Agilent Fragment Analyzer systems

Sizing, Resolution, and Purity

Introduction

Reliable and robust quality control (QC) analysis is essential throughout in vitro transcription (IVT) mRNA workflows, including vaccine development and therapeutics. Providing consistent QC minimizes risk during process development and production. Throughout the IVT mRNA vaccine workflow, there are many opportunities for QC to help aid in the development of a consistent product. The Agilent Fragment Analyzer systems can be utilized for several of these QC steps at different checkpoints in the mRNA vaccine development workflow (Figure 1)[1]. QC steps that can be performed by 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 integrity of the final IVT mRNA vaccine product.

Best handling practices for analysis of IVT mRNA with the Fragment Analyzer systems have been described[5]. This technical overview examines the ability of the systems to accurately assess IVT mRNA sizing, resolution, and percent purity.

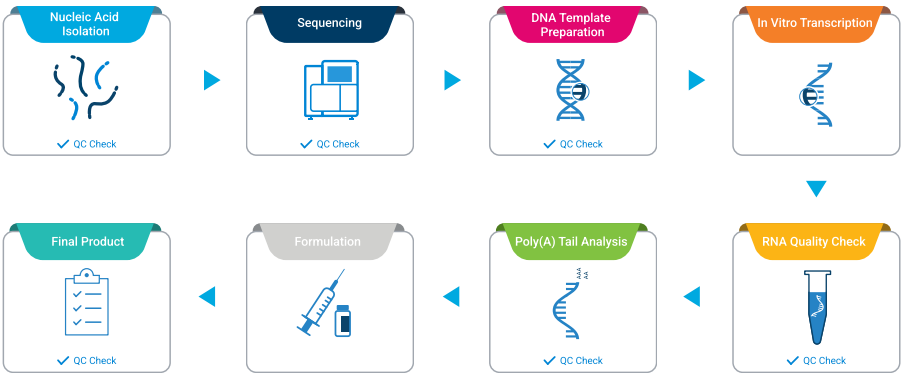


Figure 1. IVT RNA workflow with QC steps where the Agilent Fragment Analyzer systems can be used.

Experimental

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

The purified PCR products were then used as DNA templates for in vitro transcription. One microgram of each template was used to prepare an IVT mRNA sample using the T7 RiboMAX Express Large Scale RNA Production System (Promega p/n P1320). The IVT mRNA fragments were purified using the RNA Clean & Concentrator-5 kit (Zymo p/n R1013). The final samples were quantified using NanoDrop, and the size and purity were assessed using the Agilent Fragment Analyzer system with the Agilent RNA kit (15 nt) (p/n DNF-471)[6].

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

Reaction #	Sequence size (bp)
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

Sizing

To investigate the ability of the Fragment Analyzer to accurately size IVT mRNA, serial dilutions of several samples of various sizes from approximately 200 to 4,000 nt were prepared. Each dilution, from 15 to 60 ng/µl, was analyzed in triplicate using the RNA kit. Figure 2 shows a representative example of a 2,055 nt IVT mRNA, with each concentration overlaid to demonstrate consistent sizing across different concentrations. The average peak size of each of the different-sized samples at three different concentrations is shown in Figure 3. The reported size stayed consistent at each concentration tested, from 15 to 60 ng/µl.

The kit specification for IVT mRNA sizing accuracy is 10% and sizing precision 5 %CV. Here, 10 samples of varying sizes between 200 and 4,000 nt were tested at three different concentrations in triplicate. All samples displayed a sizing percent error of 10% or less, and a %CV of 1 or less across the dilution series (Figure 3). The percent error indicates good accuracy compared to the expected size across the sizing range of the kit. The low %CV is well below the kit specifications, indicating excellent precision between replicates for each size and concentration tested.

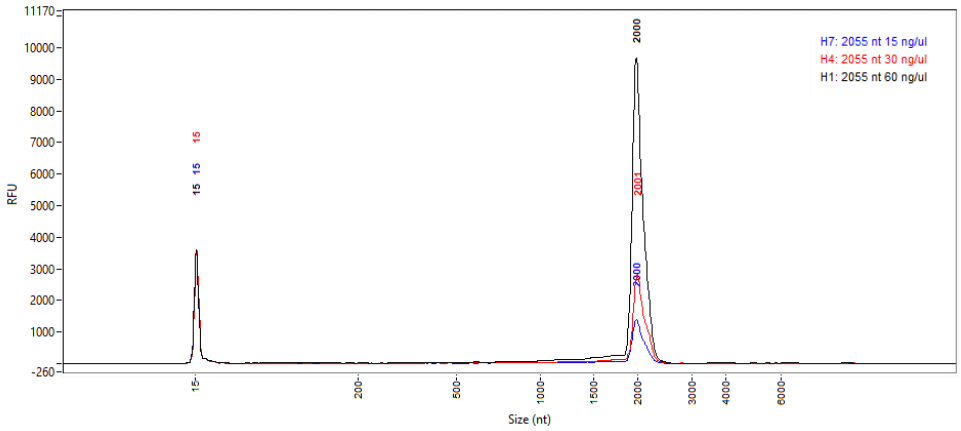
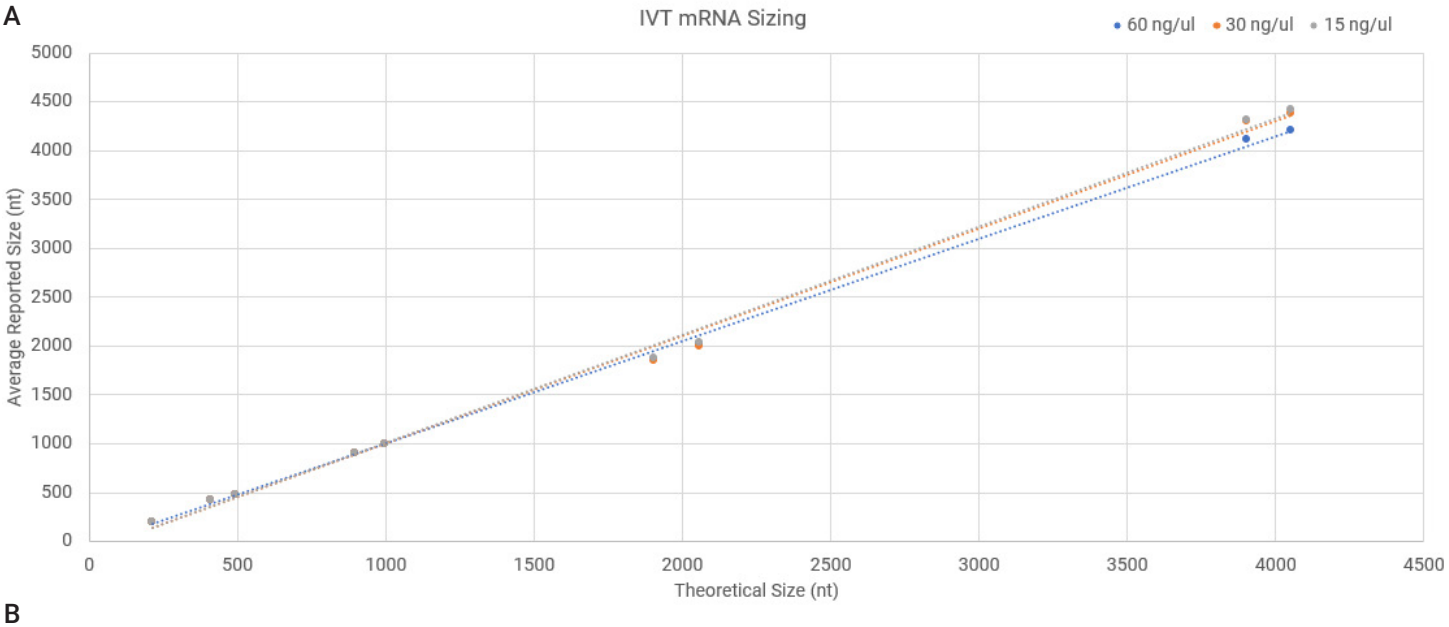


Figure 2. Representative electropherogram overlay of an IVT mRNA sample at 2,055 nt

A



B

	60 ng/µl			30 ng/µl			15 ng/µl		
Theoretical Size (nt)	Average Size (nt)	% Error	%CV	Average Size (nt)	% Error	%CV	Average Size (nt)	% Error	%CV
212	197	7.23	0.29	196	7.39	0.29	197	7.23	0.29
410	425	-3.74	0.27	425	-3.66	0.24	428	-4.47	0.55
493	481	2.43	0.42	479	2.84	0.42	481	2.43	0.29
894*	906	-1.34	0.00	904	-1.16	0.17	907	-1.49	0.05
996	1,005	-0.90	0.34	1,000	-0.44	0.06	1,005	-0.90	0.28
1,902	1,864	2.02	0.36	1,858	2.30	0.59	1,884	0.93	0.39
2,055	2,029	1.25	0.33	2,005	2.45	0.36	2,038	0.81	1.00
3,900*	4,111	-5.40	0.19	4,303	-10.33	0.93	4,319	-10.74	1.09
4,053	4,216	-4.02	0.58	4,385	-8.18	0.92	4,423	-9.12	1.07

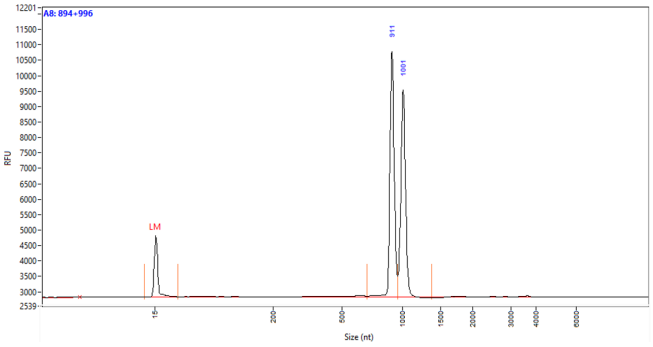
Figure 3. A) Average peak size of all IVT mRNA samples analyzed on the Agilent Fragment Analyzer system with the RNA kit. B) Table of each concentration tested. The % error and %CV at each concentration was well within the specifications of the kit. n = 3 replicates for each size and concentration tested (*n = 2).

Resolution

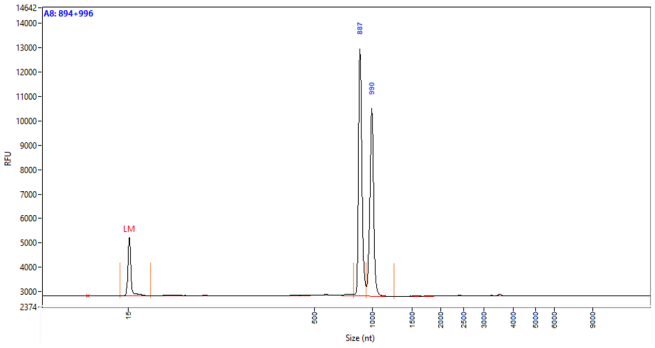
Separation resolution with the Fragment Analyzer has been examined in previous studies to demonstrate the ability of the system to separate two DNA fragments close in size and define the differing degrees of separation that can be seen in electropherograms[7,8]. In this study, IVT mRNA samples of different known sizes were analyzed both individually and mixed together to examine the resolution capabilities of the system for IVT mRNA. For example, an 894 and a 996 nt fragment, a size difference of 10.2%, showed complete baseline resolution (Figure 4A), with two distinct peaks being picked by the data analysis software algorithm. In a second example, 1,902 and 2,055 nt IVT mRNA (a 7.4% difference) samples showed limited resolution. Two peaks were detected in this size range but were not completely baseline resolved.

The Extended Method has been optimized for enhanced separation resolution of closely sized fragments[3,6]. For example, while the 1,902 and 2,055 nt mix showed only limited resolution with the normal method (Figure 4C), the fragments were partially resolved with the extended method (Figure 4D).

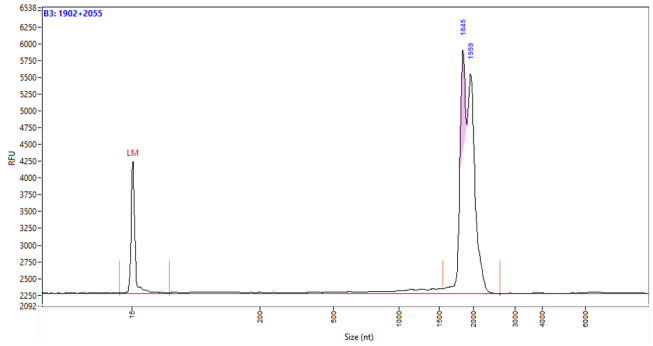
A. 894 nt + 996 nt mix. Regular method



B. 894 nt + 996 nt mix. Extended method



C. 1,902 nt + 2,055 nt mix. Regular method



D. 1,902 nt + 2,055 nt mix. Extended method

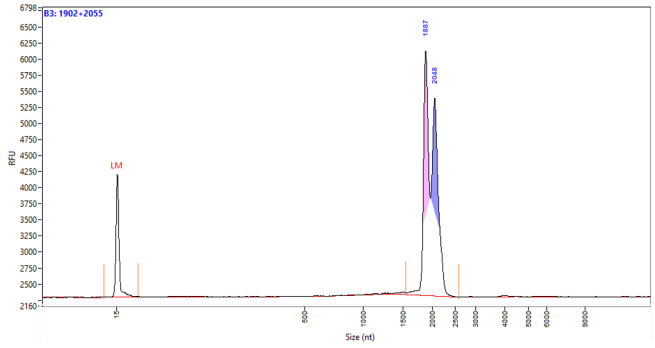


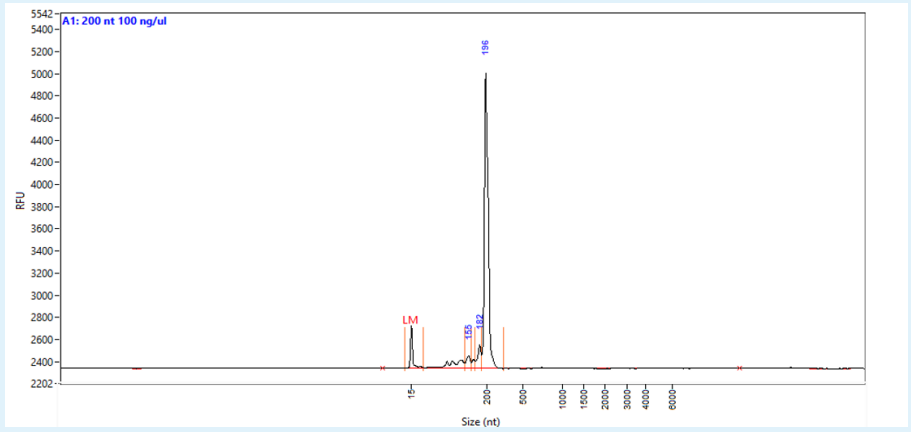
Figure 4. Resolution of the Agilent Fragment Analyzer system for IVT mRNA samples. Fragments of 894 and 996 nt were completely resolved with both A) the RNA method and B) the extended method. C) The 1,902 and 2,055 nt fragments displayed limited resolution with the regular method, but analysis with the D) extended method improved resolution of the fragments.

IVT mRNA integrity

An important aspect of IVT mRNA quality analysis is determining the integrity of the sample. To appropriately determine the percent purity of a sample, data analysis methods must be able to differentiate the main peak from any impurities or degradation. The Agilent ProSize data analysis software that is used with the Fragment Analyzer has methods that automatically detect sample peaks and report the size, concentration, and percent total of the integrated peaks. A user can also adjust settings such as the peak width and height to ensure that the entire sample is properly integrated, and to allow for the most accurate data calculations. For example, Figure 5A shows the electropherogram, Peak Table, and smear analysis table for an IVT mRNA sample that was automatically analyzed using the methods peak analysis settings, as shown. In the electropherogram, the sample is displayed as a single large peak at 196 nt, with several small peaks smearing to the left. However, the software only detects two of these peaks, at 155 and 182 nt. Changing the Minimum Peak Height from 50 to 10 RFU allows for more appropriate integration of the entire sample, with seven sample peaks being detected (Figure 4B). Further, with the standard method, the percent concentration (used to indicate the percent purity of the integrated sample peaks) of the 196 nt peak changed from 89.3 to 80.8%, giving a more accurate representation of the integrity of the sample.

The software also has a smear analysis function that can be utilized to determine the average size, concentration, and percent total of a user-defined region instead of a single peak. A smear analysis region can be set by defining a set base pair range and can be further adjusted using the mouse to grab and pull the dashed red lines, as shown in Figure 5C. In a smear analysis, the data calculations are based on the total area

A



Peak Table | Smear Analysis

	Size (nt)	ng/uL	% (Conc.) (ng/uL)	nmole/L
1	15 (LM)	0.2466		46.6452
2	155	1.4862	4.2	30.2107
3	182	2.2714	6.5	39.2646
4	196	31.2423	89.3	483.7802
	TIC:	34.9999	ng/uL	
	TIM:	553.2556	nmole/L	
	Total Conce	38.7424	ng/uL	

Total RNA Exclusion Region | Advanced Flag | Inclusion Region | Advanced Settin
Peak Analysis | Marker Analysis | Quantification | Smear Analysis | Flag

Peak Analysis
Peak Width (sec) 6
Min. Peak Height (RFU) 50
Extra Valley Points 3
Valley to Valley Baseline? ☑

Analysis Region
Start (min) 0 End (min) 0

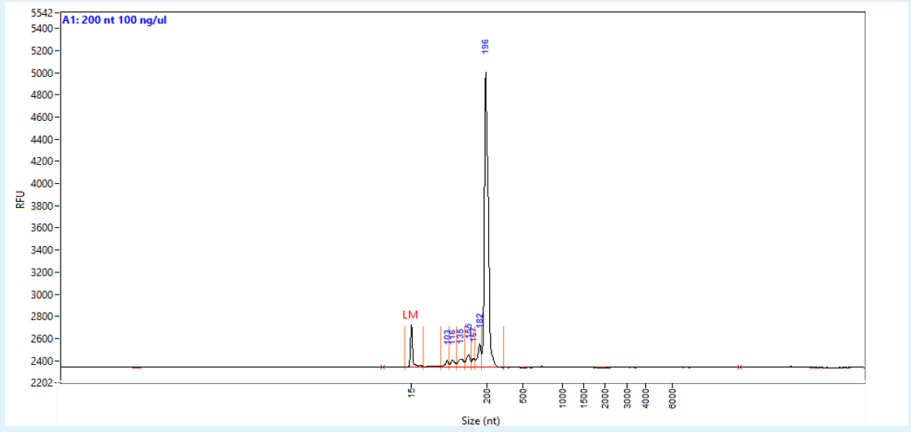
Filter Selection
Filter Binomial
# of Points 9

Manual Baseline Setpoints
Start (min) 18 End (min) 38

Peak Table | Smear Analysis

ID	Range	ng/uL	% Total	nmole/L	Avg. Size	%CV
A1: 200 nt 100 ng/ul	186 nt to 291 nt	31.3358	80.9	486.0460	201	5.37

B



Peak Table | Smear Analysis

	Size (nt)	ng/uL	% (Conc.) (ng/uL)	nmole/L
1	15 (LM)	0.2466		46.6452
2	103	0.6177	1.6	19.1792
3	116	0.8968	2.3	24.0196
4	135	1.3477	3.5	30.8068
5	155	1.4862	3.8	30.2107
6	167	0.8139	2.1	15.1611
7	182	2.2714	5.9	39.2646
8	196	31.2423	80.8	483.7802
	TIC:	38.6761	ng/uL	
	TIM:	642.4221	nmole/L	
	Total Conce	38.7424	ng/uL	

Total RNA Exclusion Region | Advanced Flag | Inclusion Region | Advanced Settin
Peak Analysis | Marker Analysis | Quantification | Smear Analysis | Flag

Peak Analysis
Peak Width (sec) 6
Min. Peak Height (RFU) 10
Extra Valley Points 3
Valley to Valley Baseline? ☑

Analysis Region
Start (min) 0 End (min) 0

Filter Selection
Filter Binomial
# of Points 9

Manual Baseline Setpoints
Start (min) 18 End (min) 38

Peak Table | Smear Analysis

ID	Range	ng/uL	% Total	nmole/L	Avg. Size	%CV
A1: 200 nt 100 ng/ul	186 nt to 291 nt	31.3358	80.9	486.0460	201	5.37

under the curve of the sample line and above the red baseline, instead of only the integrated peaks. For example, as shown in Figure 5A-B, the concentration and percent total do not change, regardless of the number of peaks that are integrated.

The concentration of the sample may impact the percent purity calculations, as too high of a concentration can cause overloading, and too low may make the impurity peaks impossible to detect, and difficult to differentiate from background noise[5]. To demonstrate this, the percent total of the 212 nt IVT mRNA sample was analyzed across a serial dilution. The peaks in each sample were integrated as discussed, and the percent total averaged across three replicates for concentration. As shown in Figure 5D, the percent total at a sample concentration of 100 ng/µl is on average 78.6%. At concentrations of 6.25 to 50 ng/µl, the percent remains consistently at about 66%. Lower concentrations result in more variability in the percent, as the smaller impurity peaks are no longer detected. The optimal concentration range for analysis with the Fragment Analyzer is thus ~ 6 to 50 ng/µl with these samples. For the most accurate analysis of IVT mRNA on the Fragment Analyzer, it is recommended to optimize the input concentration for different sample types, and ensure all samples are loaded onto the instrument at the same concentration[5].

The Fragment Analyzer was used to calculate the percent purity of a variety of IVT mRNA samples across the sizing range of the RNA kit. Each sample was analyzed across three individual capillaries in parallel at 50 ng/µl to demonstrate the reproducibility of the system (Figure 5E). At each size tested, the standard deviation of the purity was less than 1.2 and the %CV was less than 1.9%, indicating excellent precision between the replicates.

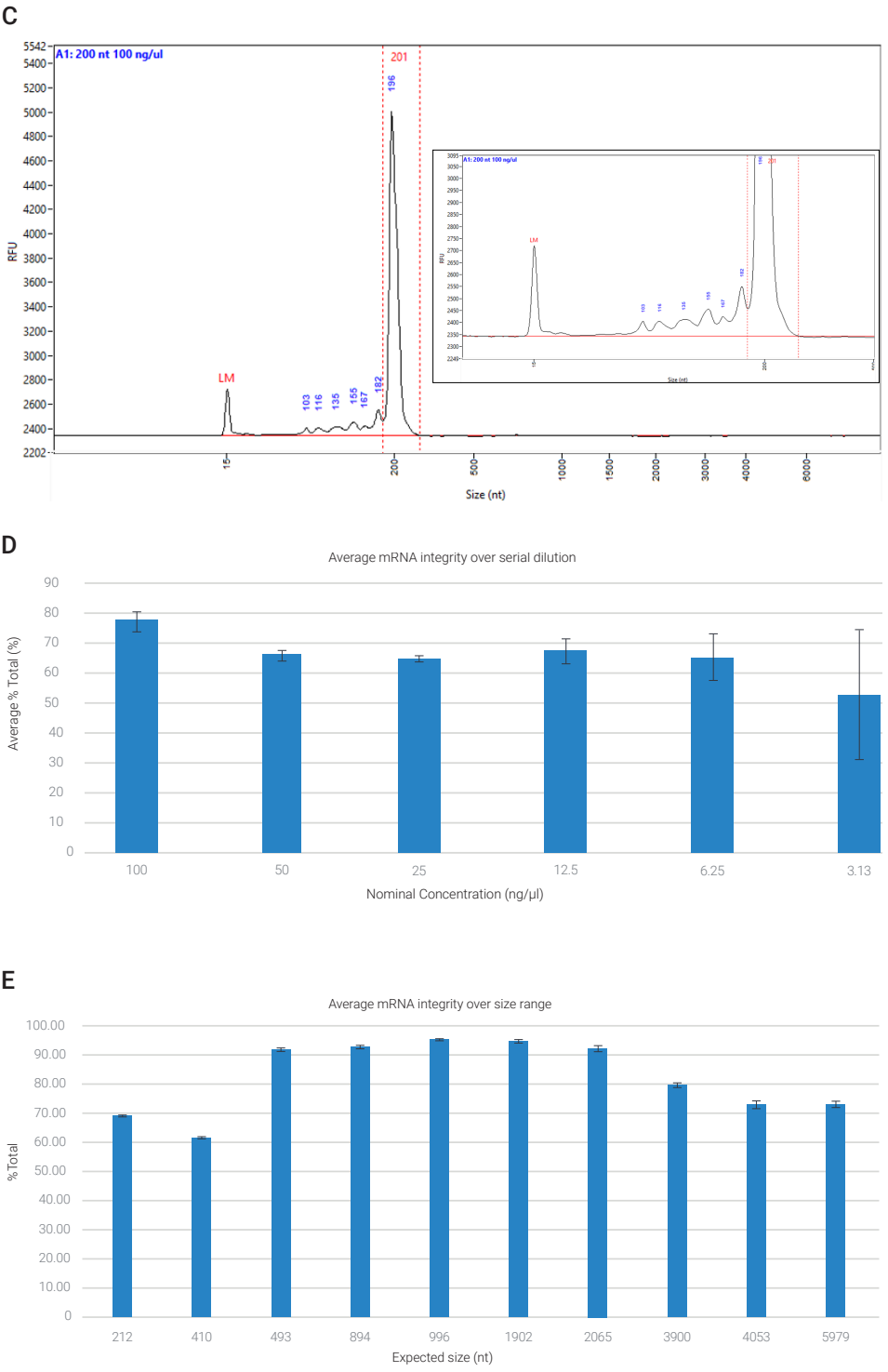


Figure 5. Best practices for integrating samples for purity analysis with the Agilent Fragment Analyzer system and Agilent ProSize data analysis software. An IVT RNA sample was analyzed with A) standard Peak Analysis settings, as shown, and B) with the minimum peak height decreased from 50 to 10 RFU. The resulting electropherograms, Peak Tables, and Smear Analysis Tables are shown. C) Smear analysis is performed by adjusting location of the red dashed lines. D) Purity across serial dilution of a 212 nt IVT mRNA. E) Average mRNA integrity of samples across the sizing range of the kit, with error bars representing standard deviation and indicating the high reproducibility at each size (n = 3).

Controlled mRNA integrity assessment

To examine the ability of the Fragment Analyzer to accurately assess mRNA integrity, a series of controlled experiments in which a smaller IVT mRNA sample was spiked into a larger sample at known concentrations was performed. The percent total of each fragment was calculated using smear analysis ranges specific to each fragment. In the example shown in Figure 6, a 1,902 nt IVT mRNA sample was kept at a constant concentration, and an 894 nt sample was added at varying amounts from 1.5 to 20% of the concentration. The smear ranges are shown in Figure 6A, with the first smear range encompassing the smaller impurity peak, and the second smear range encompassing the main fragment. The average percent total of each peak is shown in Figure 6B. It is important to note that the main fragment is not 100% pure, and that even at 0% spike-in, the purity of the sample is 87%. The percent total of the spiked-in fragment matches expectations from 1.5 to 20%. The percent total of the main fragment, indicative of the percent purity, decreases in direct correlation with the amount of smaller fragment spiked-in.

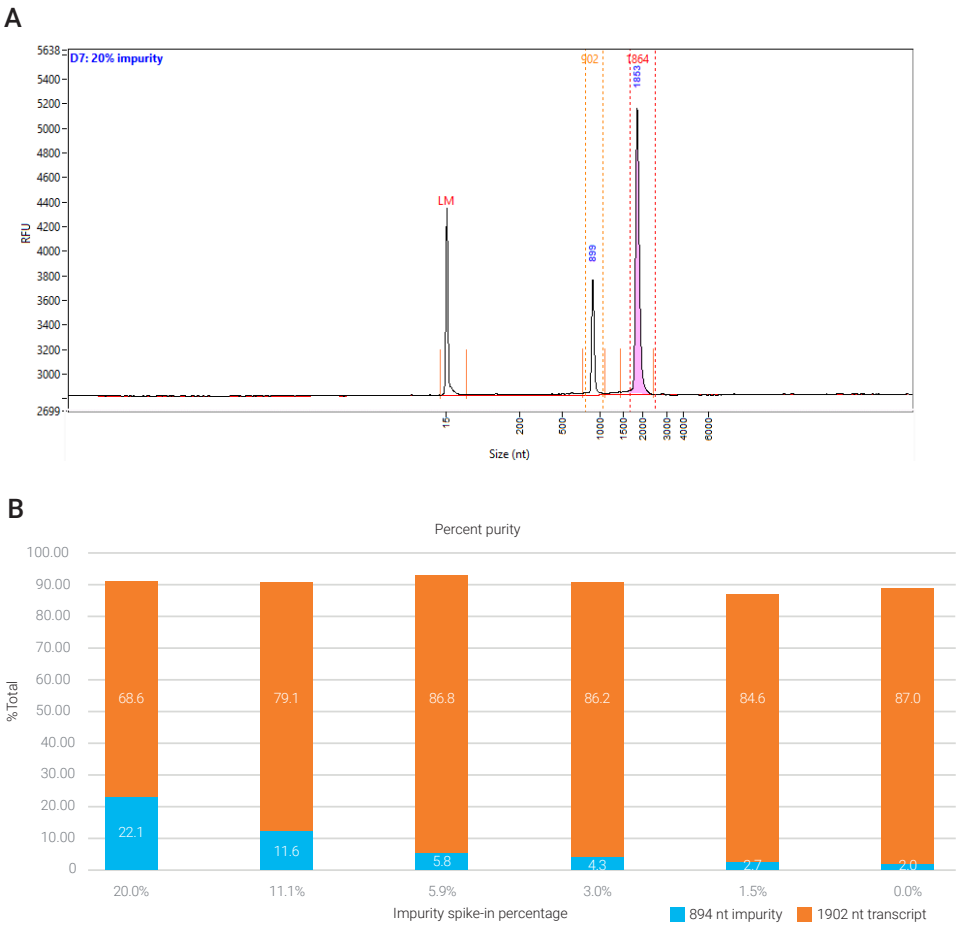


Figure 6. Controlled impurity experiments. An 894 nt IVT mRNA was intentionally added to a 1,902 nt sample at known concentrations, and analyzed on the Agilent Fragment Analyzer system. A) An example electropherogram of a 20% impurity spike-in is shown. B) The percent total of the 894 nt spiked-in impurity peak is shown in blue. The percent total of the 1,902 nt peak, indicative of the total integrity of the sample, is shown in orange.

Degradation series

To further investigate IVT mRNA integrity analysis on the Fragment Analyzer system, a 2,055 nt sample was intentionally heat degraded and analyzed at different time points. The electrophoretic profile of the sample as it is degraded is shown in Figure 7. The intact sample is displayed as a single peak, with some slight peak broadening at the base. As the sample is degraded for longer amounts of time, the peak height decreases, and a smear to the left of the peak becomes more substantial. The smear analysis range for each sample was determined by adjusting the dashed lines in the electropherograms to flank the main fragment, excluding the smear. The average percent total for each sample among the degradation is shown in Figure 7E. The percent purity recorded by the Fragment Analyzer directly correlated with the amount of time the samples underwent heat degradation.

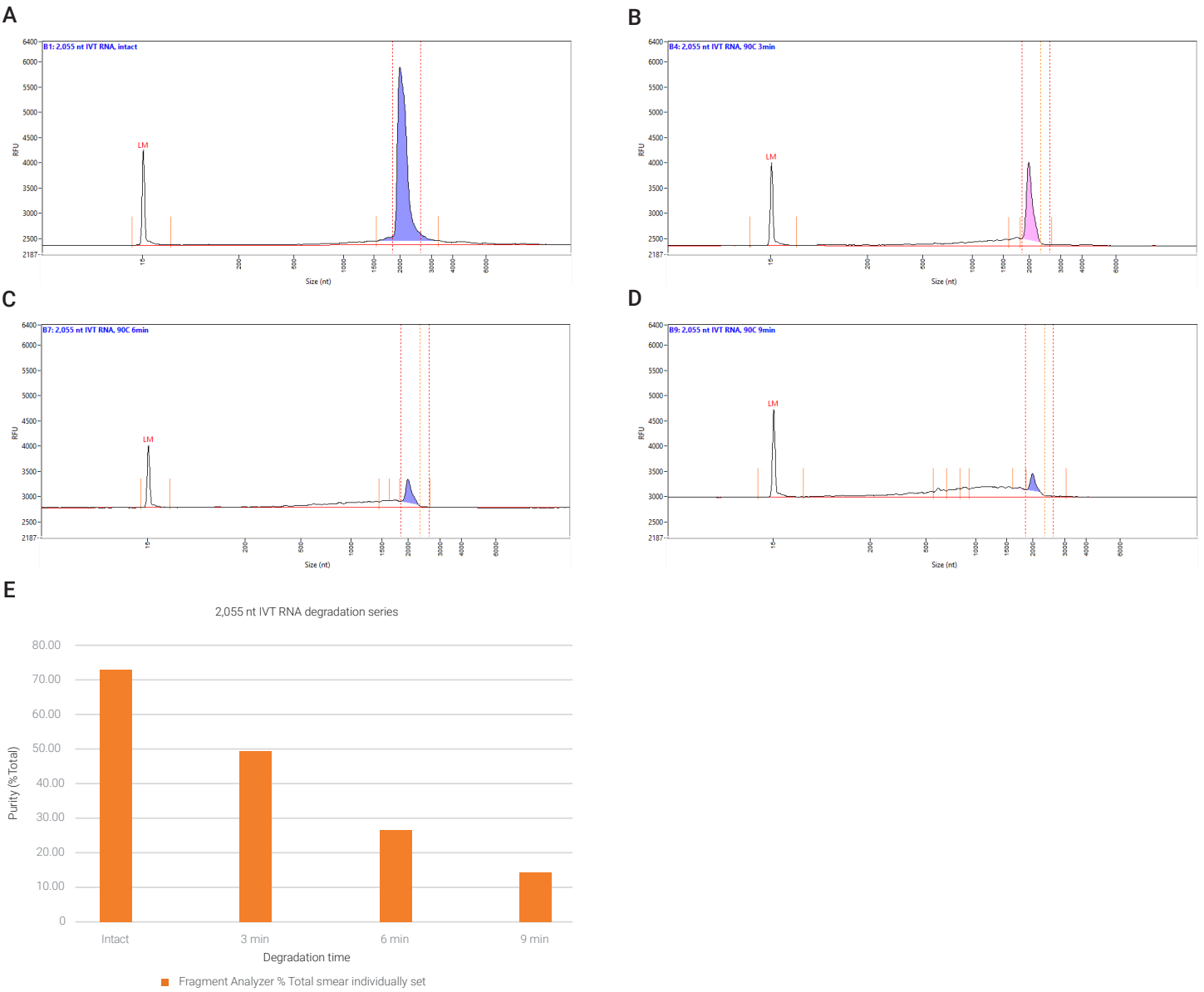


Figure 7. A 2,055 nt IVT mRNA sample was intentionally heat degraded and assessed for purity on the Agilent Fragment Analyzer system. Electropherogram images of the A) intact sample and after B) 3 minutes, C) 6 minutes, and D) 9 minutes of heat degradation demonstrate the decreasing peak heights of the sample as it is further degraded. E) The percent total of each peak decreased from an average of 73% purity for intact sample to 14% following 9 minutes of heat degradation.

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 integrity assessment. This technical overview highlights the accurate and precise sizing, high resolution, and reliable purity analysis that can be achieved with the Fragment Analyzer for IVT mRNA.

References

1. Quality Assurance in In Vitro Transcribed (IVT) RNA Vaccine Development using Agilent Automated Electrophoresis Instruments. *Agilent Technologies white paper,* publication number 5994-4760EN, **2022.**
2. Benefits of Quality Control in the IVT RNA Workflow Using the Agilent 5200 Fragment Analyzer System. *Agilent Technologies application note,* publication number 5994-0512EN, **2019.**
3. Assessment of Long IVT mRNA Fragments with the Agilent 5200 Fragment Analyzer System. *Agilent Technologies application note,* publication number 5994-0878EN, **2019**.
4. Analyzing Poly(A) Tails of In Vitro Transcribed RNA with the Agilent Fragment Analyzer System. *Agilent Technologies application note,* publication number 5994-5325EN, **2022**.
5. Best Practices for Analysis of In Vitro Transcription RNA Using the Agilent Fragment Analyzer systems. *Agilent Technologies application note,* publication number 5994-5927EN, **2023**.
6. Agilent DNF-471 RNA Kit (15 nt) Quick Guide for the Fragment Analyzer Systems. *Agilent Technologies quick guide,* publication number SD-AT000131, **2022**.
7. Highly Resolved Separation of DNA Fragments on the Agilent 5200 Fragment Analyzer System. *Agilent Technologies application note,* publication number 5994–0517, **2019**.
8. Separation Resolution Capabilities of the Agilent ZAG 135 and 110 dsDNA Kits and the Agilent Fragment Analyzer dsDNA 935 and 915 Reagent Kits. *Agilent Technologies application note,* publication number 5994–3638, **2021**.

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