Application Note

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Assessment of Long IVT mRNA Fragments with the Agilent Fragment Analyzer Systems

Authors

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Abstract

The use of *in vitro* transcription (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, quantification, and quality assessment of IVT mRNA greater than 6,000 nt was obtained using the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) and an extended RNA method.

Introduction

The synthesis of IVT mRNA results in many different types of RNA including viral RNA, mRNA, aptamer, dsRNA, CRISPR gRNA, riboprobe, and miRNA with endless application possibilities. Conventionally synthesized RNA fragments are less than 100 nt in length, but research in gene structure and functional studies requires RNA longer than 3,000 nt. In addition, RNA is prone to degradation due to factors including structure, light, heat, and RNases. The ability to detect low amounts of RNA degradation is important to the success of diverse RNA applications. The parallel capillary electrophoresis instrumentation portfolio from Agilent Technologies provides sensitive and reliable solutions for assessing varied lengths of RNA constructs[1]. The Fragment Analyzer system with the RNA kit (15 nt) provides reliable sizing and quality assessment of short and long IVT RNA.

Experimental

The experiments in this study were performed with a 5200 Fragment Analyzer system and can be replicated with comparable results on Agilent 5300 and 5400 Fragment Analyzer systems.

Separation method of RNA transcripts

IVT mRNA was produced using the RiboMAX Large Scale RNA Production System (Promega, #P1300). RNA transcripts 9,000 and 10,000 nt in length were diluted in nuclease-free water. The diluted transcripts were separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) (p/n DNF-471) with the following modifications.

The Agilent RNA Ladder (p/n DNF-382-U020) was replaced with the Lonza RNA marker 500, 1,000, 1,500, 2,000, 2,500, 3,000, 4,000, 5,000, 6,000, and 9,000 nt (Lonza, #50575) (long RNA Ladder). The stock of long RNA Ladder was diluted in nuclease-free water to 96 ng/µL, and the concentration confirmed with the Qubit 4.0 using the RNA HS assay kit (Thermo Fisher Sci., #Q32852). The long RNA Ladder was added to the Agilent RNA Diluent Marker (15 nt) (p/n DNF-369-0004) at the same ratio as described in the RNA kit protocol (2 µL ladder to 22 µL RNA Diluent Marker).

The standard separation method (8 kV for 45 minutes) for the RNA kit (15 nt) was manually altered in the Agilent 5200 Fragment Analyzer software (extended RNA method) for fragments greater than 6,000 nt to improve the resolution of high molecular weight fragments. The extended RNA method has a lower separation voltage (4 kV) and longer separation time (90 minutes) providing better separation of impurities from fragments greater than 6,000 nt (Table 1).

The extended RNA method with the long RNA Ladder was employed for all subsequent runs described within this Application Note.

Degradation series

A 100 µL sample of the 9,000 nt IVT mRNA (100 ng/µL) was heat denatured in a thermocycler at 70 °C. 12 µL aliquots were collected every two minutes, for 16 minutes, from the PCR tube and placed on ice until further analysis. The degradation series was then analyzed according to the separation method previously described.

Capped versus uncapped

A 1,800 nt IVT mRNA was transcribed using the RiboMAX kit (Promega, #P1711) with and without 5' capping and separated with the described extended RNA method.

Table 1. The Agilent RNA kit (15 nt) standard and extended method protocol for the Agilent 5200 Fragment Analyzer system.

	Agilent RNA Kit (15 nt)	
	Sample Injection	Separation Method
Standard Method	5 kV, 4 seconds	8 kV, 45 minutes
Extended Method	5 kV, 4 seconds	4 kV, 90 minutes

Results and discussion

Comparison of the normal and extended RNA method

As IVT mRNA transcripts become longer, sizing ladders need to be extended and analysis methods re-evaluated to provide the best possible results. The 5200 Fragment Analyzer system with the RNA kit (15 nt) provides accurate sizing up to 6,000 nt for IVT RNA. To accommodate accurate sizing of longer IVT mRNA, a ladder with appropriate length fragments is required. The Lonza RNA marker (long RNA Ladder) is suitable for sizing IVT mRNA above 6,000 nt due to the additional number of fragments: 500, 1,000, 1,500, 2,000, 2,500, 3,000, 4,000, 5,000, 6,000, and 9,000 nt and was used in place of the Agilent RNA Ladder (15 nt). Longer fragments require extended separation time, which aids in achieving the best resolution possible. The RNA kit (15 nt) separation method was manually adapted in the 5200 Fragment Analyzer system software (extended RNA method) to facilitate separation of IVT mRNA longer than 6,000 nt and provide better resolution of impurities from the peak of interest.

The long RNA Ladder was diluted to 96 ng/µL and separated on the 5200 Fragment Analyzer system with the RNA kit (15 nt) standard method and the extended RNA method (Figure 1). Both methods provided similar separation pattern of all 10 ladder fragments. The long RNA Ladder separated with the extended method displayed enhanced resolution compared to the standard method as seen by the increased spacing between the ladder peaks and the increased sharpness of the 9,000 nt peak. The enhanced resolution achieved with the extended method aids in the detection of minor degradation, contamination, or sizing differences from incomplete transcription.

Sizing with the long RNA Ladder was

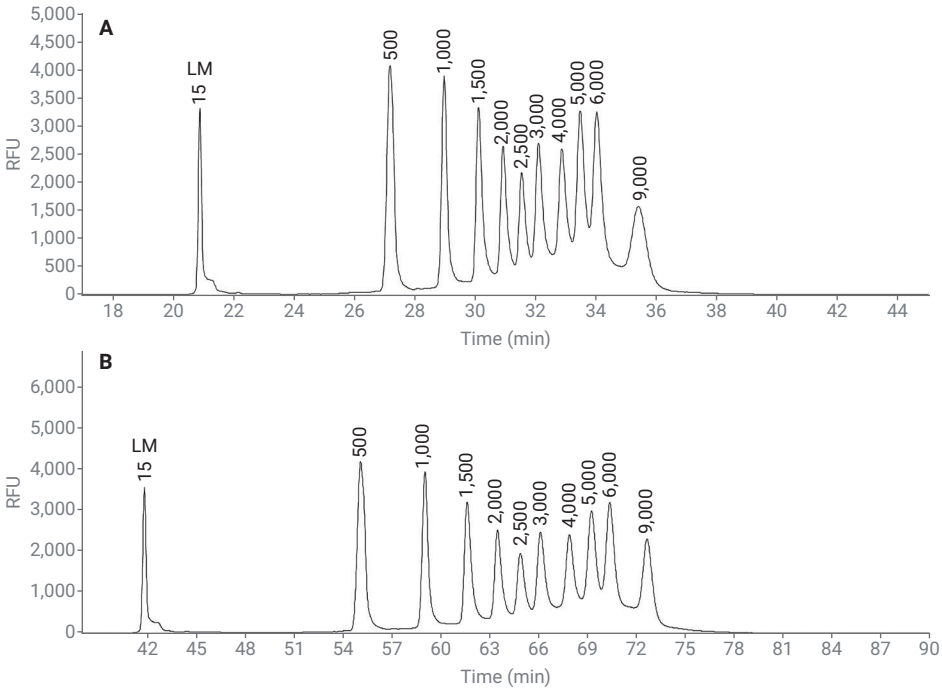

Figure 1. The long RNA Ladder separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) (A) normal method (8 kV, 45 minutes) (B) extended RNA method (4 kV, 90 minutes). LM = lower marker.

assessed with the 9,000 and 10,000 nt IVT mRNA transcripts. The RNA kit (15 nt) provides reliable quantitation for a single RNA fragment with a concentration range from 1 to 100 ng/µL. To reflect this, a dilution series of each sample over the entire concentration range of the kit (100, 50, 10 and 1 ng/µL) was assessed on the 5200 Fragment Analyzer system with the RNA kit (15 nt) standard RNA method (Figure 2) and the extended RNA method (Figure 3). Both methods reported precise sizing as indicated by the low percent coefficient of variance (% CV) throughout the dilution series (Table 2). In addition, a low percent error throughout the dilution series for both samples and with both methods indicated accurate sizing. The extended RNA method reported a lower average percent error (−0.7 %) over the entire concentration range compared to the standard RNA method (8.4 %) for the 9,000 nt sample. The 10,000 nt IVT mRNA average percent sizing error was similar between the two separation methods. The extended RNA method is recommended when extremely accurate sizing or high resolution is needed for determining the presence of degradation or sizing differences from incomplete transcription in IVT mRNA samples longer than 6,000 nt.

Degradation series

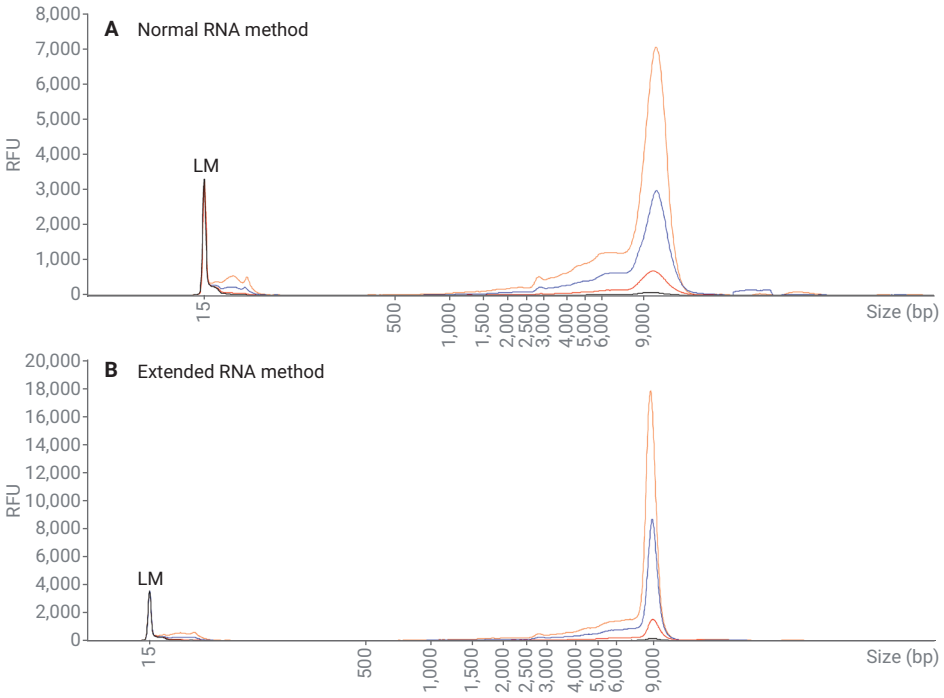

Figure 2. Overlay of the 9,000 nt IVT mRNA fragments over a dilution series of 100, 50, 10, 1 ng/µL separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) using the long RNA Ladder and (A) the normal RNA method (B) the extended RNA method. LM = lower marker.

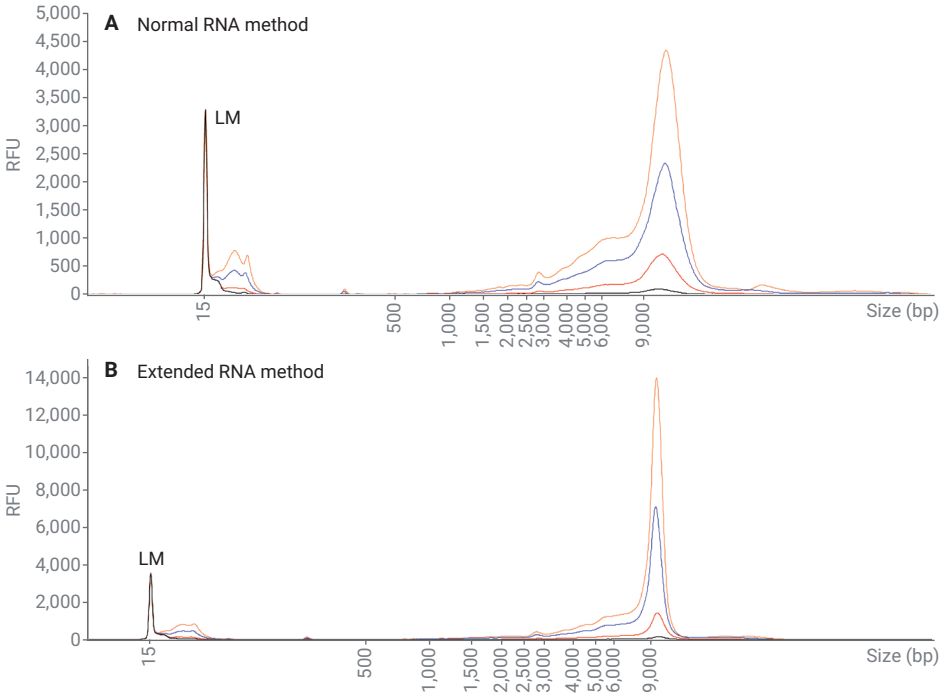

Figure 3. Overlay of the 10,000 nt IVT mRNA fragments over a dilution series of 100, 50, 10, 1 ng/µL separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) using the long RNA Ladder and (A) the normal RNA method (B) the extended RNA method. LM = lower marker.

The smear analysis feature in the Agilent ProSize data analysis software can be used to determine the percent of degradation from the DNA template, and the percent of IVT mRNA. The 9,000 nt IVT mRNA was degraded at 70 °C for 0 to 16 minutes. Every two minutes an aliquot was taken and placed on ice. The degradation series was separated on the 5200 Fragment Analyzer system with the RNA kit (15 nt) using the extended RNA method and long RNA Ladder (Figure 4). The electropherogram and gel image displayed a decrease in the expected peak height with increased degradation time. Smear analysis was performed to determine the percent of degradation (smear range 75 to 7,500 nt) and the percent of the peak of interest (smear range 7,500 to 11,000 nt). The percent concentration was recorded for each smear range and compared between time points. As expected, the percent degradation increased with incubation time, in correlation with a decreased percent of the peak of interest as the sample degraded (Table 3). A narrow smear analysis range of the peak of interest can help to identify varying sizing from incomplete transcription. The smear analysis feature offers a simple approach for assessing sample quality.

Capped versus uncapped

Table 2. IVT RNA analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) and separated with both the normal RNA method and the extended RNA method (A) 9,000 nt sample (B) 10,000 nt sample. *n=3.

A	Sizing Throughout Dilution Series					
Concentration (ng/µL)	Theoretical Size 8,989 nt					
	Normal RNA Method			Extended RNA Method		
	Average* (nt)	% CV	% Error	Average* (nt)	% CV	% Error
100	9,910	1.6 %	10.2 %	8,705	0.9 %	-3.2 %
50	9,862	0.8 %	9.7 %	9,051	2.3 %	0.7 %
10	9,718	0.6 %	8.1 %	8,977	0.4 %	-0.1 %
1	9,491	0.4 %	5.6 %	8,971	0.7 %	-0.2 %
1 to 100	9,745	0.8 %	8.4 %	8,926	0.8 %	-0.7 %

B	Sizing Throughout Dilution Series					
Concentration (ng/µL)	Theoretical Size 10,003 nt					
	Normal RNA Method			Extended RNA Method		
	Average* (nt)	% CV	% Error	Average* (nt)	% CV	% Error
100	10,736	0.2 %	7.3 %	9,453	1.3 %	-5.5 %
50	10,676	0.3 %	6.7 %	9,501	1.1 %	-5.0 %
10	10,496	0.4 %	4.9 %	9,597	0.8 %	-4.1 %
1	10,197	0.8 %	1.9 %	9,620	0.2 %	-3.8 %
1 to 100	10,526	0.4 %	5.2 %	9,543	0.5 %	-4.6 %

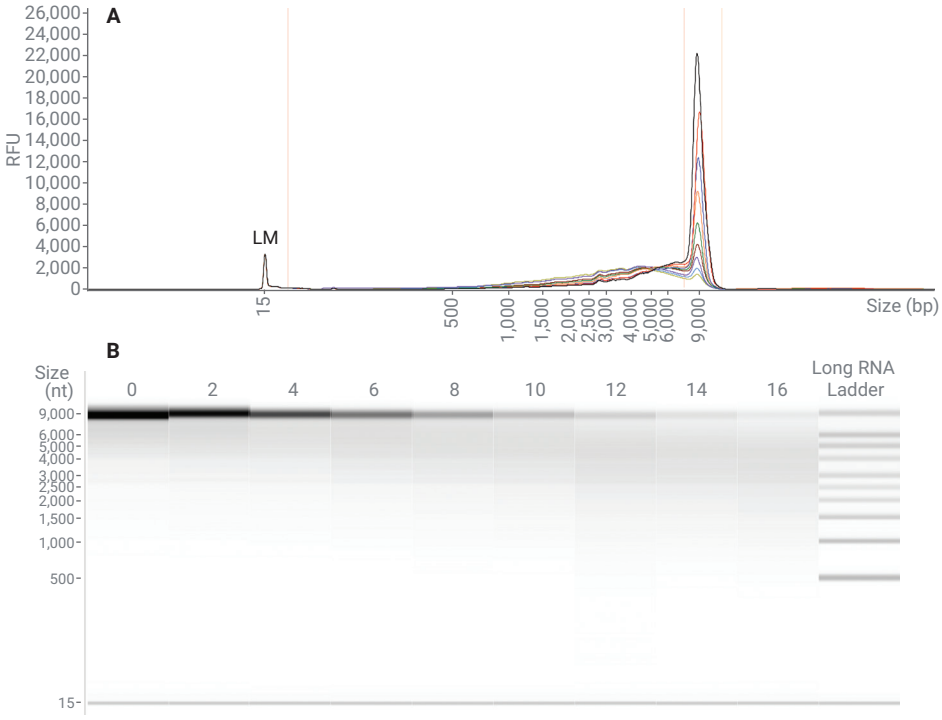

Figure 4. 9,000 nt IVT mRNA analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) using the long RNA Ladder and extended RNA method over a degradation series at 70 °C from 0 to 16 minutes. (A) Electropherogram overlay of degradation series. (B) Gel image of degradation. LM = lower marker.

A common modification made to IVT mRNA transcript is the addition of a 7-methylguanylate cap at the 5' end. A 5' cap stabilizes the RNA transcript, prevents degradation, and assists in binding during translation. Capped and uncapped 1,800 nt IVT mRNA were analyzed on the 5200 Fragment Analyzer system with the RNA kit (15 nt) using the extended RNA method and long RNA Ladder (Figure 5). The extended RNA method was used to provide the best resolution possible. An average size of 1,810 nt was observed for both the capped and uncapped RNA transcripts. RNA capping did not influence the sizing or general profile of the transcript.

Conclusion

The Agilent 5200 Fragment Analyzer system offers reliable quality control analysis essential to IVT mRNA workflows. Accurate sizing of IVT mRNA greater than 6,000 nt was achieved with the Agilent RNA kit (15 nt) using a long RNA Ladder with both the normal and extended RNA method. The extended RNA method offers the option of exceptional sizing to confirm complete transcription and the ability to distinguish minute amounts of degradation. The ProSize Smear Analysis tab in the Agilent ProSize data analysis software enables the simultaneous detection of the percent of the peak of interest and the percent of degradation. This feature aids the user in quickly identifying a quality sample. In addition, the 5200 Fragment Analyzer and the RNA kit (15 nt) provided accurate sizing with the addition of a 7-methylguanylate cap at the 5' end of the RNA transcript.

Table 3. Smear analysis of the percent concentration for two regions of the 9,000 nt IVT mRNA throughout the degradation series separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) using the extended RNA method and the long RNA Ladder. *n=2.

Smear Analysis of Degradation Series 9,000 nt IVT RNA		
70 °C Incubation Time (minutes)	Percent of Degradation (75 to 7,500 nt)*	Percent of Peak of Interest (7,500 to 11,000 nt)*
0	36.8 %	62.7 %
2	44.6 %	54.9 %
4	52.3 %	46.7 %
6	60.8 %	36.5 %
8	70.0 %	28.1 %
10	77.3 %	20.9 %
12	85.3 %	13.4 %
14	87.7 %	10.4 %
16	90.5 %	7.5 %

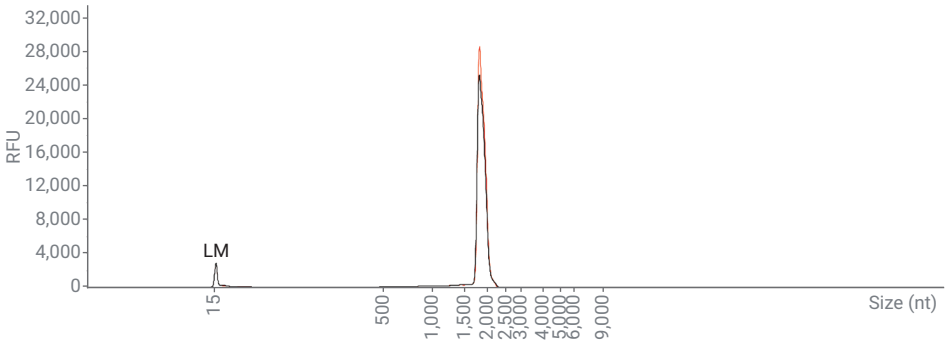

Figure 5. Overlay of the 1,800 nt IVT mRNA fragments capped (red) and uncapped (black) separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) using the extended RNA method and the long RNA Ladder. LM= lower marker

Reference

1. Warzak, D.; Pocernich, C.; Wong, K-S. Benefits of Quality Control in the IVT RNA Workflow Using the Agilent 5200 Fragment Analyzer System, *Agilent Technologies Application Note*, publication number 5994-0512EN, **2018**.

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